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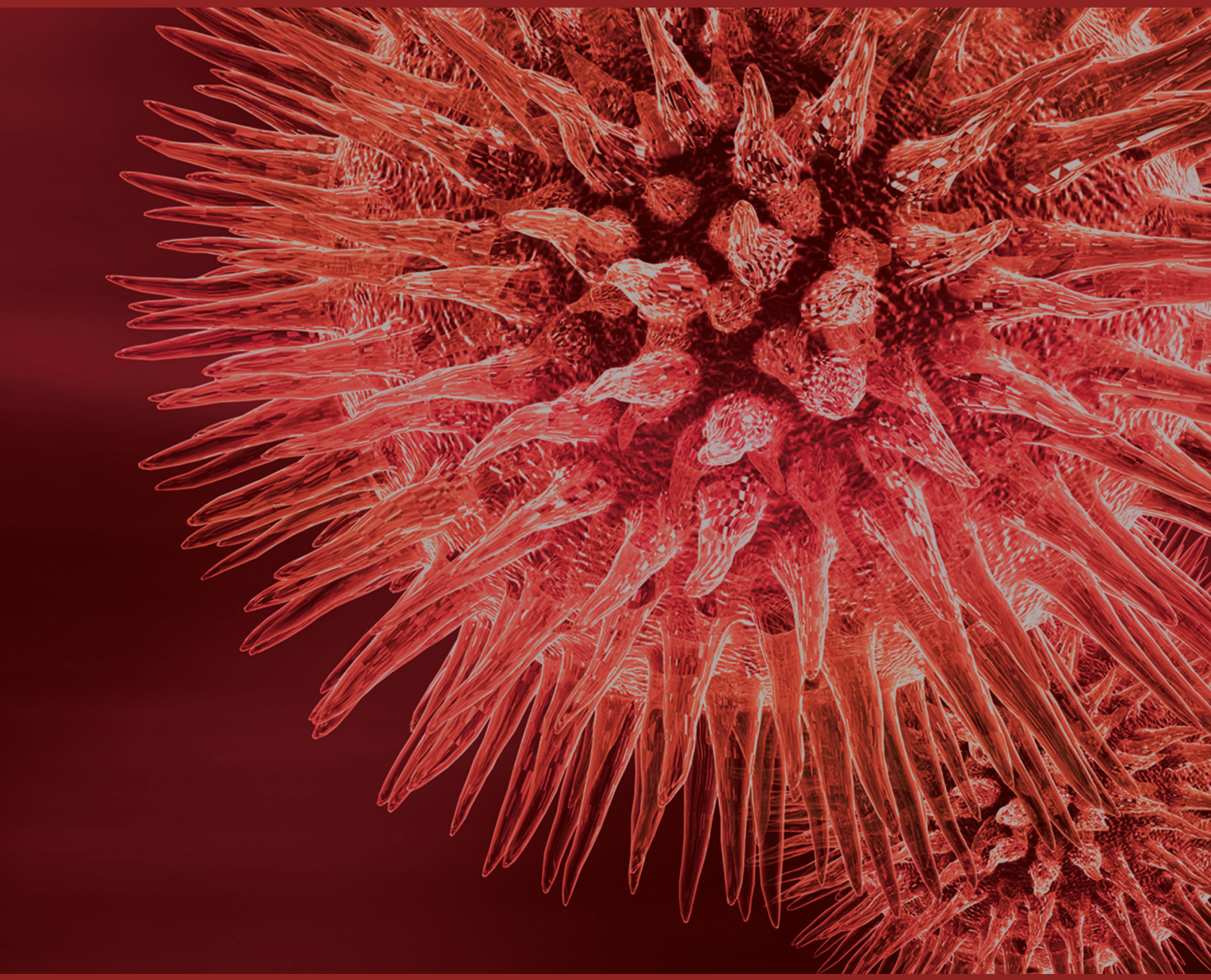
Guest Editors: Spyros S. Skandalis, Katalin Dobra, Martin Götte, Evgenia Karousou, and Suniti Misra



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Editorial

Impact of Extracellular Matrix on Cellular Behavior: A Source of Molecular Targets in Disease

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In the last few decades, outstanding strides have been made on understanding the impact of extracellular matrices (ECMs) on cellular behavior in health and disease. It is now clear that ECMs are not inactive space-filling materials but, in contrast, apart from their structural roles they interact with cells and generate signals to control a multitude of vital cellular functions. Currently, there are strong indications that ECMs could potentially play a groundbreaking role in drug discovery since they comprise an invaluable source of multiple molecular targets. The established key roles of specific ECM effectors, such as proteoglycans, hyaluronan (HA), biosynthetic enzymes, cytokines, and growth factors, in the development and progression of several diseases suggest that ECMs hold a great potential in driving the design and development of novel disease targeting tools.

The focus of this special issue is to highlight the role and impact of specific ECM effectors on cellular behavior as well as their potential targeting that could advance the treatment of various diseases, such as skeletal and skin disorders, fibrosis, and cancer. It consists of two original research papers and four review articles covering a broad range of topics.

S. Albeiroti et al. in their paper entitled "Hyaluronan's Role in Fibrosis: A Pathogenic Factor or a Passive Player?" point out critical parameters that link HA to fibrosis and discuss the role of HA as well as its cellular receptors and HA anabolic/catabolic enzymes in different fibrotic diseases. The presented data suggest that HA and its regulatory pathways potentially represent novel targets for antifibrotic therapies.

A. Korpetinou et al. in their paper entitled "Increased Expression of Serglycin in Specific Carcinomas and Aggressive Cancer Cell Lines" evaluate the expression of the proteoglycan serglycin in several cancer cell lines and tissues and find that serglycin is expressed at high levels in more aggressive cancers. This experimental study suggests that the overexpression of serglycin by cancer and stromal cells may augment the expression of inflammatory mediators and proteases affecting the behavior of both stromal and cancer cells and providing a novel molecular target in aggressive cancers.

M. A. Soares et al. in their article entitled "Heparan Sulfate Proteoglycans May Promote or Inhibit Cancer Progression by Interacting with Integrins and Affecting Cell Migration" discuss the role of the interplay between integrins and heparan sulfate proteoglycans (such as syndecans and basement membrane proteoglycans) in health and cancer progression. This review highlights the need of further analysis and deeper understanding of the functions of integrin-heparan sulfate proteoglycans interactions in cancers in order to develop novel treatments based on analog molecules or prognostic factors that would be beneficial to patients.

The review by S. Mizumoto et al. entitled "Mutations in Biosynthetic Enzymes for the Protein Linker Region of Chondroitin/Dermatan/Heparan Sulfate Cause Skeletal and Skin Dysplasias" focuses on the recent advances in the study of cartilage and connective tissue disorders caused by defects in the biosynthesis of the common glycosaminoglycan-protein linker region tetrasaccharide in proteoglycans, called glycosaminoglycan linkeropathies. The authors describe the mutations of the glycosyltransferases responsible for the biosynthesis of the linker region and suggest that a deeper understanding of the molecular pathogeneses of glycosaminoglycan linkeropathies may lead to the design of new therapeutics for these diseases.

The review by B. Gidwani and A. Vyas entitled "A Comprehensive Review on Cyclodextrin-Based Carriers for Delivery of Chemotherapeutic Cytotoxic Anticancer Drugs" summarizes the advantages of the cyclodextrin-based nanotechnology for effective delivery of anticancer drugs aiming at minimizing off-target effects observed with other drug delivery systems.

H. Pratsinis and D. Kletsas in their paper entitled "Organotypic Cultures of Intervertebral Disc Cells: Responses to Growth Factors and Signaling Pathways Involved" use a 3D culture system in order to study intervertebral disc (IVD) degeneration. They investigate the response of IVD cells in 3D organotypic gels to growth factors as well as the signaling pathways involved and propose that these culture systems may be useful for the design of novel regenerative therapies of degenerated IVD.

In conclusion, several emerging issues related to the critical roles of ECMs molecules in the regulation of cell behavior are presented in this special issue. We hope that this issue will add to the rapidly expanding field of matrix pathobiology underlying specific diseases and will help in the development of matrix-based therapeutic strategies in the near future.

Acknowledgments

We would like to thank all the pioneers in the matrix biology research field who brought forth the key roles of ECMs in health and disease and contributed to major developments in this area. We are very grateful to all the authors for their informative contributions and the reviewers for their support and constructive critiques in making this special issue possible. We would also like to express our appreciation to Professor Nikos Karamanos for valuable discussions and suggestions during the editing of this special issue.

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Review Article

Hyaluronan's Role in Fibrosis: A Pathogenic Factor or a Passive Player?

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Fibrosis is a debilitating condition that can lead to impairment of the affected organ's function. Excessive deposition of extracellular matrix (ECM) molecules is characteristic of most fibrotic tissues. Fibroblasts activated by cytokines or growth factors differentiate into myofibroblasts that drive fibrosis by depositing ECM molecules, such as collagen, fibronectin, and connective tissue growth factor. Transforming growth factor-β (TGF-β) is one of the major profibrotic cytokines which promotes fibrosis by signaling abnormal ECM regulation. Hyaluronan (HA) is a major ECM glycosaminoglycan that is regulated by TGF-β and whose role in fibrosis is emerging. Aside from its role as a hydrating, space filling polymer, HA regulates different cellular functions and is known to have a role in wound healing and inflammation. Importantly, HA deposition is increased in multiple fibrotic diseases. In this review we highlight studies that link HA to fibrosis and discuss what is known about the role of HA, its receptors, and its anabolic and catabolic enzymes in different fibrotic diseases.

1. Introduction

(1) Extracellular Matrix Remodeling in Fibrosis. A significant volume of living tissues is occupied by extracellular space that is filled with the extracellular matrix (ECM). Molecules of the ECM, fibrous proteins, and glycosaminoglycans (GAGs) are organized at the surface of the cells that produce them and form a network between cells [1]. Aside from its classical role in structure and support, the ECM has a major role in cell regulation, migration, proliferation, and survival. While ECM remodeling is very important in the normal development, many studies have also linked the remodeling of ECM to disease pathogenesis and, notably, to fibrosis [2]. Fibrosis, the formation of scar tissue that results from excessive aberrant wound healing, is characterized by a significant increase in ECM deposition. Inflammation is known to be a major contributing factor to fibrosis. Whereas mild inflammation ends in the restoration of normal tissue architecture, severe and chronic inflammation result in tissues losing their ability to heal. This promotes a fibrogenic repair response, the excessive accumulation of ECM, and the formation of abnormal tissue architecture. Subsequently, sustained fibrosis leads to impaired organ function [3, 4].

Fibrosis is driven primarily by myofibroblasts. Fibroblasts are normally classified as major ECM-producing cells in the human body. When activated, fibroblasts differentiate into a myofibroblastic cell type that expresses alpha smooth muscle actin (α-SMA) and overexpress collagen and fibronectin [5, 6]. Fibroblast activation, proliferation, differentiation, and migration initiate the fibrotic process. Fibroblasts can be activated by many inflammatory mediators, among which are tumor necrosis factor (TNF-α), platelet-derived growth factor (PDGF), interleukin-33, interleukin-13, and transforming growth factor beta (TGF-β), the well-studied and major mediator of fibrosis that acts through SMAD-dependent and SMAD-independent signaling pathways [4, 7–10].

After tissue injury, TGF-β levels are significantly increased, which aids in recruiting immune cells, like neutrophils and macrophages, and activating fibroblasts, which release further TGF-β. TGF-β mediates the deposition of many ECM proteins, including collagen and fibronectin, and matricellular proteins (ECM-bound proteins that have regulatory and signaling roles but not structural roles) such as connective tissue growth factor (CTGF) [11]. Through the action of SMAD proteins, TGF-β promotes collagen deposition both by enhancing the expression of different

types of collagen genes and by mediating the overexpression of collagenase inhibitors [12]. TGF-β also promotes the expression and deposition of another major ECM protein, fibronectin, specifically the extra domain A- (EDA-) containing fibronectin. EDA-fibronectin has a major role in myofibroblast differentiation and wound healing and its expression is upregulated during tissue repair and scar formation [13]. Interestingly, Bhattacharyya et al. have recently shown that EDA-fibronectin is significantly increased in mice with bleomycin-induced cutaneous fibrosis and, importantly, is a ligand for toll-like receptor- (TLR-) 4. The group also showed that, *in vitro*, EDA-fibronectin treatment stimulated myofibroblast differentiation and collagen production and the effect was blocked by blocking TLR4 signaling [14]. A third major mediator of tissue remodeling and fibrosis that is stimulated by TGF-β is CTGF. CTGF is a matricellular protein that is known to interact with different cytokines and cellular receptors as well as with other ECM proteins and is expressed only during the process of wound repair. Not only is CTGF overexpressed during scar formation, but it is also required for persistent TGF-β-driven fibrosis, as seen in a mouse fibrosis model where CTGF inhibition has been shown to prevent and reverse fibrosis [15].

In addition to the abovementioned ECM proteins that are involved in fibrogenesis, proteoglycans and GAGs have also been shown to be involved in this process. Evidence shows that the deposition of versican, a chondroitin sulfate-containing proteoglycan, is significantly increased in the ECM of lung lesions from patients with idiopathic pulmonary fibrosis [16]. In bleomycin-induced pulmonary fibrosis in rats, the increased levels of TGF-β associated with a significant increase in biglycan mRNA and a decrease in decorin mRNA, both of which are classified as small leucine-rich proteoglycans [17]. Another report has shown, in a rat model of a bleomycin-induced pulmonary fibrosis, that levels of versican, heparin sulfate, and fibromodulin are increased in fibrotic lungs [18]. Importantly, the same group has shown that alterations in proteoglycan and GAGs are associated with alterations in the viscoelastic properties of lung parenchymal tissues early in the fibrotic response [19]. This change in the viscoelastic properties of the lung during fibrosis could partially explain the impaired function of the organ. Additionally, alterations in the expression of enzymes implicated in synthesis and sulfation of GAGs have been reported in fibrosis. Fibroblasts derived from fibrotic lungs expressed increased mRNA levels of xylosyltransferase-I and chondroitin-4-sulfotransferase-I compared to their nonfibrotic counterparts. However, expression of these two enzymes was increased in the nonfibrotic lung fibroblasts in response to TGF-β stimulation via p38 mitogen-activated protein kinase (MAPK) and TGF-β type-1 receptor/activin receptor-like kinase 5 pathways [20]. Collectively, published data suggest that remodeling of ECM during fibrosis is a regulated process that involves the activity of anabolic enzymes and catabolic enzymes. An additional major ECM molecule that has an underappreciated but rapidly emerging role in the fibrotic process is hyaluronan (HA).

(2) Hyaluronan. HA is an ubiquitous, nonsulfated, unbranched GAG and the largest polysaccharide produced in vertebrates. It is the only GAG that does not have a core protein component. The HA chain is made up of repeating disaccharides; each disaccharide is composed of D-*N*-acetylglucosamine and D-glucuronic acid linked by alternating β-(1,4) and β-(1,3) glycosidic bonds [21]. Three mammalian HA synthase (HAS) enzymes have been identified: HAS1, HAS2, and HAS3; their structure is well-conserved among various mammalian species. HA is synthesized uniquely and unlike other GAGs that are synthesized in the Golgi apparatus, at the inner surface of the cell membrane by one of the HAS enzymes, where UDP-*N*-acetylglucosamine and UDP-glucuronic acid are added alternately to the reducing end of the HA chain being synthesized. The growing HA molecule then translocates extracellularly through the membrane. Studies have shown that HAS2 is responsible for the majority of HA synthesis and that HAS2 deletion in mice results in embryonic lethality due to severe cardiovascular defects [22–24].

Under many conditions, HA exists in the body bound to one of its protein partners, such as CD44, versican, and aggrecan [25]. CD44 is ubiquitously expressed glycoprotein present on most mammalian cells and is considered the major cell surface receptor for HA. The interaction between CD44's cytoplasmic tail with many intracellular proteins, including kinases and cytoskeletal components, allows for HA to exert a wide range of different cell regulatory functions [26]. CD44 also has a role in HA catabolism by assisting HA-degrading enzymes. Catabolism of HA in humans occurs by endo-β-*N*-acetylhexosaminidase enzymes known as hyaluronidases (HYALs). Out of the six discovered HYAL enzymes, HYAL1 and HYAL2 are the only somatically active HA degrading enzymes in humans [27, 28] (Figure 1). HYAL1 was identified as an acid-active enzyme in serum in 1967 by De Salegui et al. and later confirmed to be a lysosomal enzyme [29, 30]. On the other hand, HYAL2, also an acid-active enzyme, is a glycosylphosphatidylinositol- (GPI-) anchored cell-surface protein [31]. The turnover of HA occurs rapidly in the body. HA is present normally in high amounts in multiple tissues and fluids of the body, including the joints, the eye vitreous, the umbilical cord, and amniotic fluid. High levels of HA are also present in proliferating tissues and tissues undergoing repair. As a result, the rapid catabolism of HA, through the activity of HYAL enzymes, represents a major mechanism by which HA levels are regulated in the body [32]. Studies have shown that HA degradation is dependent upon the classical HA binding receptor CD44 and involves mainly HYAL1 and HYAL2 [33, 34]. In addition to the enzymatic processes that cleave HA, HA can be degraded by oxidation reactions; particularly, reactive oxygen species (ROS) and free radicals are known to degrade HA polymers [35].

(3) Hyaluronan and Inflammation. Elevated levels of accumulated HA have been observed in many inflammatory diseases. For example, high levels of HA in joint tissues of patients with rheumatoid arthritis have been reported. Additionally, multiple studies have reported increased HA deposition in inflammatory diseases of the liver. Whereas the concentration of

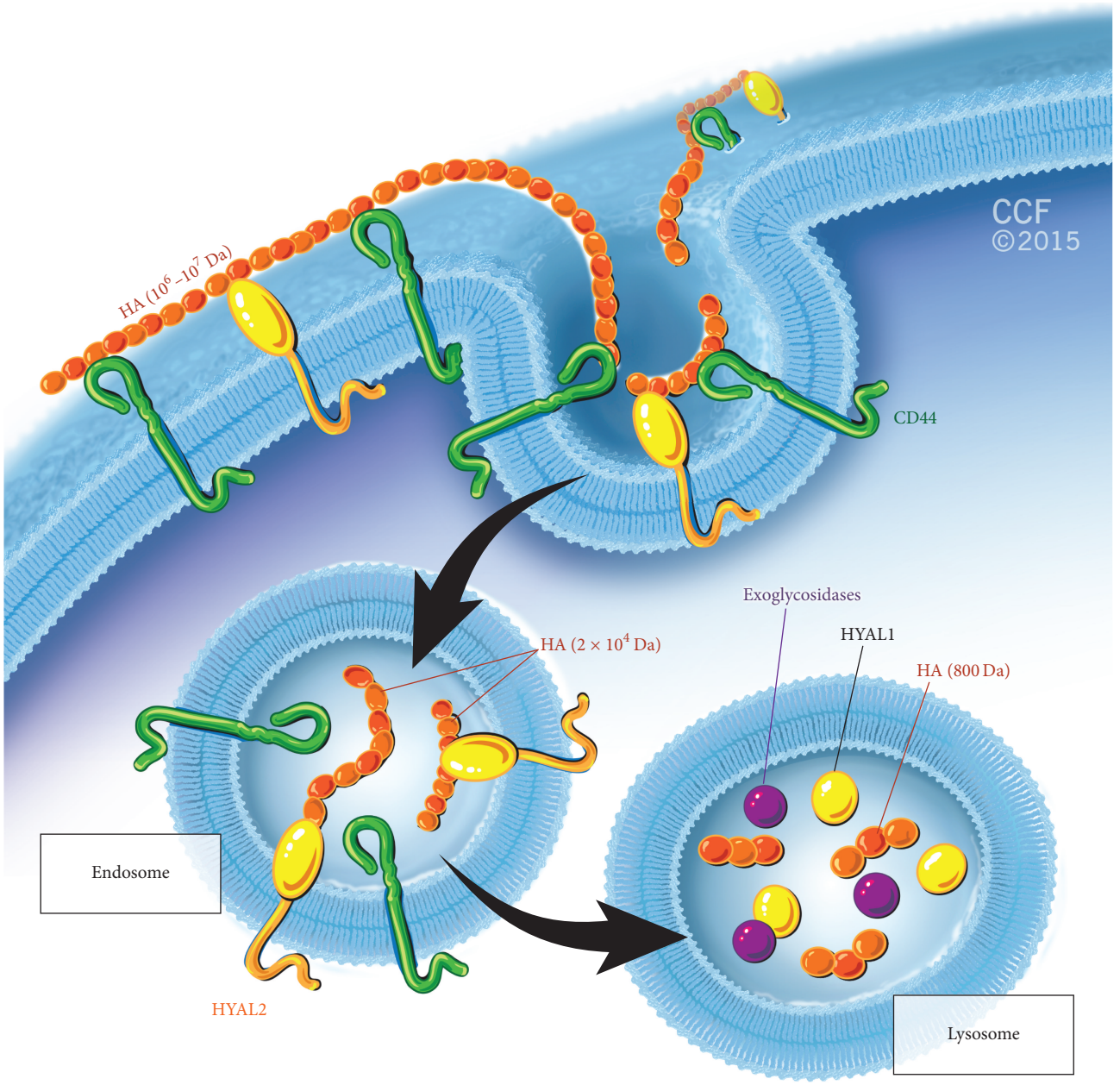
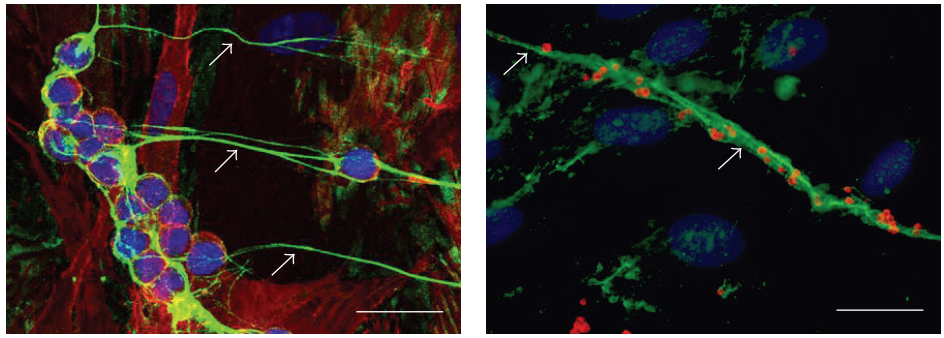


Figure 1: Schematic representation of a currently accepted model for cell-mediated HA degradation. Catabolism of HA starts at the cell surface. CD44 binds HA extracellularly and facilitates its degradation by HYAL2. The degradation products (now in the range of 20-kDa polymers) are internalized into endosomes and then transported into the lysosomes where they get further degraded by HYAL1 into tetrasaccharides. *N*-acetyl glucosaminidase and glucuronidase enzymes then further degrade HA into monosaccharides. "Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved".

HA in the healthy liver is low, its concentration significantly increases in inflamed liver, leading to increased levels of serum HA. As a result, the level of circulating HA has been proposed as a biomarker for cirrhotic liver disease, for monitoring liver function, for assessing liver fibrosis, and for diagnosing chronic viral hepatitis C [36]. HA levels also increase in patients with inflammatory bowel disease (IBD), asthma, and idiopathic pulmonary arterial hypertension [37–39].

Accumulating evidence from studies published in the last 20 years has confirmed that the molecular weight of HA is critical in determining its biochemical and cellular roles. Numerous reports have shown that different sizes of HA exert a wide spectrum of functions [40, 41]. In tissues under normal conditions, HA is present in its high molecular weight (HMW-HA) form with an average size range of 1–10 × 10^6 daltons. HMW-HA functions as a structural, hydrating



(a) (b)

Figure 2: HA cables (green) on the surface of stimulated mucosal intestinal smooth muscle cells (M-SMCs) bind monocytes (round nuclei, red = CD44) (a) and platelets (red for CD42b) (b). Cultured M-SMCs were treated with polyI:C, a double-stranded RNA that mimics a viral infection, for 18 hours at 37°C. PolyI:C-stimulated M-SMCs were then coincubated with monocytes or platelets, methanol-fixed, and histochemically stained for HA (green and white arrows) and either CD44 (a) or CD42b (b) (scale bar = 25 μm).

polymer due to its hydrophilic properties. However, HMW-HA is also known to be anti-inflammatory. Large HA polymers have a role as molecules that indicate the integrity of tissues and control the cellular inflammatory responses [42]. For example, HMW-HA can protect from T-cell-mediated liver injury and bleomycin-mediated lung injury in mice and it can promote the suppressive effects of regulatory $CD4^+$ $CD25^+$ T cells [43–45]. Conversely, reports indicate that low molecular weight HA (LMW-HA) has proinflammatory effects. LMW-HA, or HA fragments that result from degradation of intact HMW-HA, has been shown to act as Damage Associated Molecular Patterns (molecules that can mediate and perpetuate an immune response in the absence of an infectious agent). Many reports have shown that fragmented HA is capable of signaling cellular responses through specific receptors, including CD44 and toll-like receptors (TLR) 2 and 4 [41, 42]. However, recent studies have shown that HA fragments can also induce another form of innate host defense responses, via TLR4, at the intestinal epithelium [46].

Under certain pathological conditions, HMW-HA can also become proinflammatory in the form of large leukocyte-adhesive, protein-decorated cable structures. During inflammation, inter-alpha-trypsin inhibitor (IαI), a serum protein, can leak into the extravascular spaces as a result of increased vascular permeability. The exposure of IαI to the extracellular matrix allows it to function as a heavy chain donor to HA. TNF-α-stimulated gene 6 (TSG-6), an enzyme and HA binding protein, facilitates the transfer of heavy chains 1 and 2 from IαI to HA to form leukocyte-adhesive HA cables. Importantly, TSG-6 expression increases in the inflamed tissues, which emphasizes the role HA cables play in inflammation [47]. Leukocyte-adhesive HA matrices have been reported in many inflammatory diseases, including intestinal tissues of IBD patients, lung tissues of asthmatic patients, lung tissues from idiopathic pulmonary hypertension patients, and synovial fluid of patients with arthritic disease [48, 49]. The production of HA cables can also be stimulated *in vitro* by inflammatory stimuli. Figure 2 shows leukocytes (2A) and platelets (2B) bound specifically to HA cables produced by polyI:C-stimulated intestinal smooth muscle cells.

2. Hyaluronan and Fibrosis

HA plays an important role in fibrosis that has just recently become more appreciated. In the 1980s and 1990s, very few studies reported on the correlation between HA and fibrosis. However, a significantly increased number of publications have emerged in the last ten years suggesting a key role for HA in the fibrotic process, primarily in fibrotic lung and kidney. In 1989, Bjermer et al. compared HA levels in bronchoalveolar lavage (BAL) fluid from patients with idiopathic pulmonary fibrosis with those from healthy controls. The group reported that BAL HA levels in the tested patient population were five times higher than their healthy counterparts. However, serum HA levels in the idiopathic pulmonary fibrosis patients were comparable to those in the nondiseased population. Importantly, the increase in the amounts of HA in patients correlated significantly with the observed increase in BAL neutrophil and lymphocyte counts as well as the severity of the disease [50]. The same group later reported that HA levels, along with fibronectin levels, were significantly increased in the lung tissue, as well as in BAL fluid, during bleomycin-induced lung injury in rats. However, the accumulation of HA and fibronectin preceded the development of pulmonary fibrosis [51]. The data collectively suggest that HA may be playing an indirect role in promoting fibrosis: HA is known to bind and recruit immune cells and its accumulation before fibrosis suggests increased immune cell recruitment, as observed in patients with idiopathic pulmonary fibrosis. Immune cells, in turn, release a variety of inflammatory mediators and growth factors that are known to activate fibroblasts leading to fibrosis [3, 47–49]. Because HA is significantly increased in fibrotic tissues, multiple reports have suggested the use of HA levels as a biomarker for fibrosis, particularly for liver fibrosis [52, 53]. In addition, unpublished data from our lab shows increased deposition of HA in fibrotic intestines from IBD patients compared to non-IBD controls (Figure 3).

One of the first observations that TGF-β affects HA synthesis was in studies on limb development in the late 1980s. Synthesis of HA and pericellular coat formation in

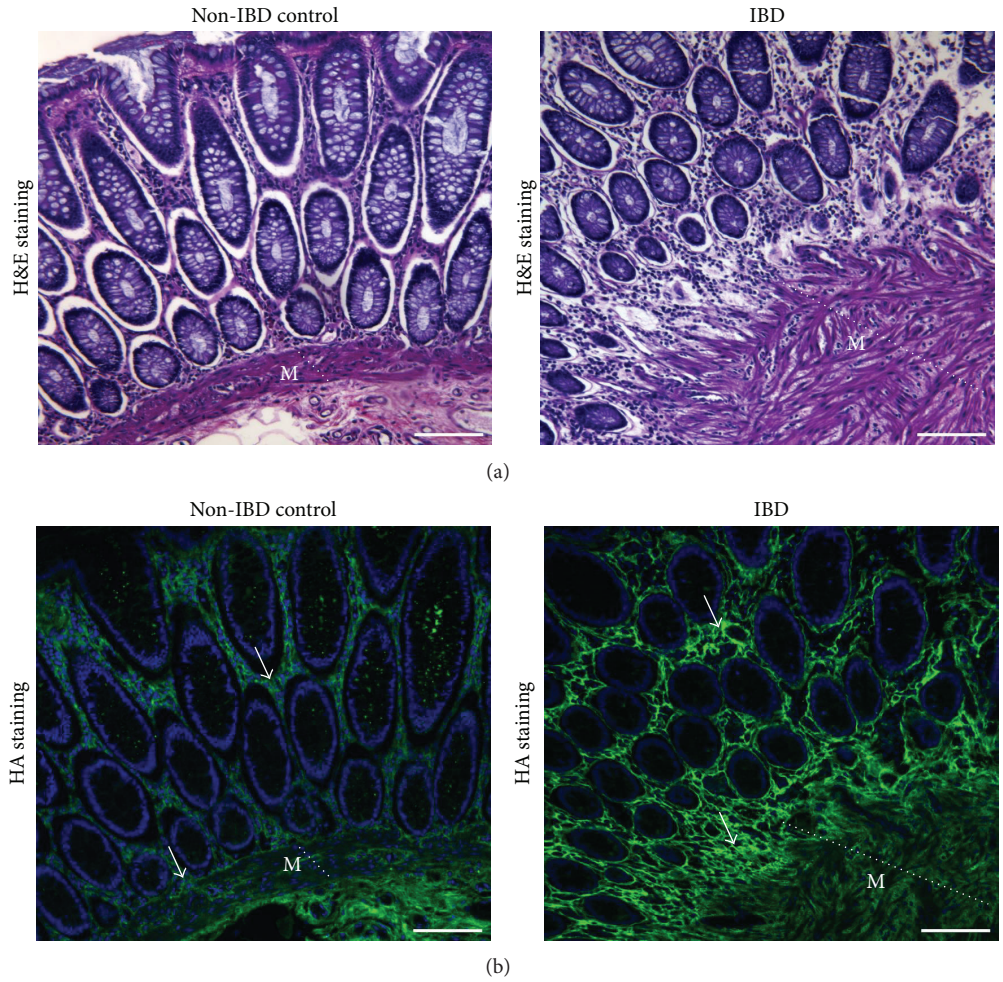


Figure 3: Colon tissue sections from a non-IBD and an IBD patient stained with hematoxylin & eosin (H&E) (a) or HA binding protein ((b), HA = green and DAPI = blue). The IBD colon shows symptoms of fibrosis, characterized by the expanded *muscularis mucosae* (dotted white line, M) compared to non-IBD control. Fluorescence histochemical staining shows elevated deposition of HA in both the epithelium and the *muscularis mucosae* as labeled by the white arrows. Scale bar (solid line) represents 100 μm. IBD is a chronic inflammatory disease of unknown etiology. Development of fibrosis is a common and serious complication of IBD, one which requires surgical intervention to repair. It is thought that fibrosis in IBD stems from the chronic nature of inflammation signaling uncontrolled levels of wound healing.

the mesoderm were stimulated by TGF-β [54]. In a concurrent study, when the effect of multiple growth factors on HA synthesis in cultured human foreskin fibroblasts was tested, not only did TGF-β stimulate HA production, but PDGF, epidermal growth factor, and basic fibroblast growth factor stimulated increased HA as well [55]. In multiple studies published in 1990, TGF-β was shown to promote HA production in cultured lung fibroblasts [56, 57]. The effect of TGF-β on HA synthesis in skin fibroblasts has also been reported. Whereas Westergren-Thorsson et al. found that TGF-β did not enhance HA production in skin fibroblasts [56], other studies have reported the opposite. For example, it was reported that TGF-β enhanced mRNA expression of HAS1 and HAS2 in cultured mouse skin fibroblasts [58]. Interestingly, Ellis and Schor reported that TGF-β inhibited HA synthesis by cultured skin fibroblasts when the cells were subconfluent, whereas it upregulated HA synthesis by confluent cells [59]. In human fibroblast-like synoviocytes, TGF-β was a strong stimulus for HAS1 transcription and HA synthase activity via a MAPK-dependent pathway, whereas it reduced HAS3 mRNA [60]. Strong evidence in the literature supports the hypothesis that TGF-β, which is a key inducer of fibrosis, also has an undeniable role in driving HA expression by fibroblasts.

One mechanism by which HA could be promoting fibrosis is through enhancing aberrant fibroblast motility. In addition to the production of HA, TGF-β also stimulates the expression of HA-mediated motility receptor (RHAMM), which, through interaction with HA, promotes cell locomotion. Samuel et al. showed that TGF-β, which is also a stimulator of motility, mediates the transcription and membrane expression of RHAMM along with HA production, resulting in an increase in motility response by cells [61]. The role of RHAMM-HA interaction in fibrogenesis was confirmed in later studies. Particularly, a peptide that specifically blocked HA-RHAMM binding but not HA-CD44 or HA-TLR binding

was able to block fibroblast migration and alter wound repair in wild-type but not RHAMM-knockout mice. Additionally, the specific blockade of HA-RHAMM interaction caused a reduction in macrophage count and fibroblast number in excisional wounds in rats and blocked RHAMM-regulated focal adhesion kinase pathways in cultured fibroblasts [62]. The data suggest that RHAMM-HA interaction-mediated fibroblast migration contributes to inflammation and fibrogenesis and that targeting this interaction presents a novel approach to treating fibrosis.

Several reports suggested a role for HA in kidney fibrosis. Ito et al. reported that the treatment of cultured proximal tubular cells with HMW-HA and LMW-HA resulted, through activation of MAPK signaling cascade, in increased cell migration in scratch-wound assays. However, HMW-HA was a more potent stimulator of cell migration compared to LMW-HA. Interestingly, the effect of HA on cell migration was abrogated by blocking CD44. The group also tested the role of endogenous HA in cell migration and found that scratch-wounded cells produced significantly higher amounts of HA compared to control cells and that blocking CD44 or MAPK reduced cell migration [63]. One possibility is that the CD44 and RHAMM pathways that enhance cell migration are related and that HA needs to interact with both receptors in order to exert its cell-migratory effect that leads to fibrogenesis. In 2010, Han et al. suggested a role for HA and its receptors during interstitial fibrosis in chronic renal injury. The group reported that expression of HA, CD44, and lymphatic vessel endothelial hyaluronan receptor- (LYVE-) 1 increased in fibrotic tissue areas and that HA accumulation was accompanied by an increase in α-SMA [64]. More evidence supporting the role of HA in renal fibrosis comes from the studies of Kato et al. on basigin, which is a transmembrane protein known to regulate matrix metalloproteinase expression and enhance the production of HA in fibroblasts. The group found that basigin-deficient mice demonstrated significantly less fibrosis than wild-type animals after induced renal injury. Importantly, embryonic fibroblasts from basigin-deficient mice expressed lower levels of HAS2 than wild-type fibroblasts. In addition, TGF-β enhanced HAS2 expression, along with α-SMA mRNA, only in wild-type fibroblasts but not in basigin-deficient cells [65]. A recently published work by Colombaro et al. confirmed the possible role of HA in renal fibrosis. The researchers used deficient mice in one of the HA-degrading enzymes, HYAL1 or HYAL2, to demonstrate the effect of the increased accumulation of HA in the kidney following renal injury. Whereas HYAL1- and HYAL2-deficient mice suffered from intensified inflammation, wild-type mice demonstrated significant reduction in renal damage. In addition to increased HA accumulation, HYAL-deficient mice expressed increased levels of CD44, α-SMA, and collagen compared to wild-type mice 30 days after renal injury [66]. Data suggested that dynamic catabolism of HA, by the HYAL enzymes, is protective against renal injury by reducing the levels of accumulated HA that can contribute to kidney inflammation and fibrosis.

Steadman and Philips have extensively investigated the role of HA in fibrosis during the last 10 years. In an interesting study comparing the response to TGF-β stimulation between dermal and oral fibroblasts, the researchers found that TGF-β, through the activity of SMAD3, induced proliferation in dermal fibroblasts whereas it inhibited proliferation in oral fibroblasts. Importantly, levels of HA released by dermal fibroblasts were significantly higher than those released by oral fibroblasts and blocking HA synthesis in dermal fibroblasts inhibited the proliferative function of TGF-β [67]. The study is important because it demonstrates that the effect of TGF-β depends on the levels of HA produced in fibroblasts; after injury, HA appears to be a key factor that contributes to TGF-β-induced scar formation, whereas in oral fibroblasts, which are known to heal without scarring and fibrosis, HA levels are low. In a follow-up study, the group showed that TGF-β-dependent fibroblast proliferation depended on the expression of CD44 and that CD44-EGFR interaction is required for the proliferative effect of TGF-β via MAPK/ERK pathway. Interestingly, the researchers were able to induce TGF-β-dependent proliferation in oral fibroblasts by overexpressing HAS2, confirming the role of accumulated HA in fibrosis [68]. In other studies, the group showed that TGF-β-dependent fibroblast-to-myofibroblast transformation is also dependent upon HA/CD44/EGFR involvement [69]. In 2015, Midgley et al. reported on the role of HA catabolism in the fibrotic process, particularly in myofibroblast differentiation. The group showed that Bone Morphogenetic Protein 7, which is a cytokine known to have antifibrotic effect, prevented TGF-β-dependent lung myofibroblast differentiation by promoting cell-surface HA internalization and degradation by HYAL2 and CD44 variant isoform CD44V7/8 [70]. The reports published by Steadman and Philips suggest collectively that targeting proteins involved in HA production or degradation represents a unique approach to the prevention and probably the reversal of fibrosis. More evidence confirming the central role of HA and CD44 in pulmonary fibrosis, particularly *in vivo*, was reported by Li et al. The researchers overexpressed HAS2 specifically in the myofibroblasts of mice and found that these animals demonstrated severe fibrosis and higher mortality than wild-type mice after bleomycin-induced lung injury. In an assay that evaluated the invasiveness of fibroblasts in a composite matrix with basement membrane constituents, the group found that fibroblasts isolated from bleomycin-treated mice invaded matrix more readily than control fibroblasts and that this invasion was dependent upon the expression of HAS2 and CD44. Importantly, the study showed that development of lung fibrosis *in vivo* was also dependent on HAS2 and CD44 expression [71].

Although the majority of reports in the literature suggest that HA promotes fibrogenesis and myofibroblast differentiation, the findings of a few researchers suggest the opposite. For example, Evanko et al. reported that HA controls fibrosis by binding to fibrillar matrix components. The group found that TGF-β treatment of lung fibroblasts resulted in the colocalization of HA and fibronectin in the ECM of the formed myofibroblasts. Importantly, inhibition of HA synthesis or disruption of ECM-HA resulted, surprisingly, in increased fibronectin and collagen deposition as well as increased α-SMA expression and myofibroblast phenotype

enhancement [72]. It is unclear why this discrepancy exists; however, it suggests that HA's role in myofibroblasts is complex and more research is needed to better understand the role of HA in fibrosis. In 2012, Li et al. took a totally novel approach to study the role of HA in fibrosis, particularly in synovial fibrosis *in vivo*. Using an osteoarthritis mouse model, the researchers tested the effect of HA injection in abrogating fibrosis and the tissue changes that result from TGF-β treatment. Interestingly, the group found that HA treatment protected against TGF-β-induced fibrosis in the wild-type animals. However, HA treatment had no protecting effect on CD44-knockout mice, suggesting a role for HA-CD44 binding in exerting the protective effects of HA [73].

It is unknown whether HMW-HA or LMW-HA has the most potent effect in driving fibrosis or whether size matters at all in the fibrotic process. Most studies on the role of HA in fibrosis were performed assuming that HA is in its native form, which is generally its HMW-HA form. One of the exceptions is the work mentioned above from Turley's laboratory on the effect of RHAMM on fibrogenesis [62]. The researchers found that LMW-HA had higher binding affinity to RHAMM than HMW-HA and predicted that HA fragments are essential promoters of fibrosis, which is also partially based on the fact that LMW-HA is well-known to be proinflammatory [42]. A possible mechanism by which LMW-HA promotes fibrosis is through driving aberrant wound healing. In one study, Tolg et al. measured the effect of HA fragments on promoting wound healing and showed that LMW-HA stimulated dermal fibroblast migration and closure of excisional wounds. Furthermore, the group found that HA fragments increased the accumulation of TGF-β1 and the infiltration of macrophages in the wound [74]. Work by other groups showed that LMW-HA stimulated increased expression and production of TGF-β3, collagen, and tissue inhibitor of matrix metalloproteinase in dermal fibroblasts and endothelial cells [75, 76]. The promotion of wound healing, production of TGF-β, and deposition of collagen by LMW-HA suggest a different mechanism by which HA can promote fibrosis, one that possibly involves inflammation. Because levels of HA in fibrotic conditions are reported to be high, more HA fragments could be created, leading to uncontrolled wound healing and, subsequently, to fibrosis.

3. Conclusion

The presented body of evidence suggests that HA is important in fibrotic repair response and investigating fibrosis in the light of HA regulation is essential. Furthermore, because the molecular weight of HA generally determines its function, investigating what role different HA sizes have in fibrogenesis might open new doors to understanding the pathogenic process that leads to fibrosis. Our previous knowledge about HA in fibrosis was limited to the earlier publications that mostly reported on a correlation between increased HA levels and fibrosis, which suggested the use of HA as a biomarker for diagnosing fibrosis. However, recent work, particularly from labs of Steadman, Philips, Turley, and Noble, clearly showed that HA, in fact, is not just a passive player or an outcome of fibrosis but rather a driving factor that is required for TGF-β to exert its profibrotic effects. HA contributes to fibrosis by mediating fibroblast motility, fibroblast proliferation, and fibroblast-to-myofibroblast differentiation. In addition, reports have linked HA receptors, particularly CD44 and RHAMM, as well as HA synthases and degrading enzymes to fibrosis. This suggests that HA and its regulation pathways potentially represent novel targets for antifibrotic therapies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Increased Expression of Serglycin in Specific Carcinomas and Aggressive Cancer Cell Lines

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In the present pilot study, we examined the presence of serglycin in lung, breast, prostate, and colon cancer and evaluated its expression in cell lines and tissues. We found that serglycin was expressed and constitutively secreted in culture medium in high levels in more aggressive cancer cells. It is worth noticing that aggressive cancer cells that harbor KRAS or EGFR mutations secreted serglycin constitutively in elevated levels. Furthermore, we detected the transcription of an alternative splice variant of serglycin lacking exon 2 in specific cell lines. In a limited number of tissue samples analyzed, serglycin was detected in normal epithelium but was also expressed in higher levels in advanced grade tumors as shown by immunohistochemistry. Serglycin staining was diffuse, granular, and mainly cytoplasmic. In some cancer cells serglycin also exhibited membrane and/or nuclear immunolocalization. Interestingly, the stromal cells of the reactive tumor stroma were positive for serglycin, suggesting an enhanced biosynthesis for this proteoglycan in activated tumor microenvironment. Our study investigated for first time the distribution of serglycin in normal epithelial and cancerous lesions in most common cancer types. The elevated levels of serglycin in aggressive cancer and stromal cells may suggest a key role for serglycin in disease progression.

1. Introduction

Proteoglycans are composed of a specific core protein substituted with one or more covalently linked glycosaminoglycan chains. Proteoglycans are either secreted in the extracellular matrix or are located at the cell membrane and intracellularly [1]. They participate in the organization of extracellular matrix but also regulate cell phenotype and properties in tissues [2]. Proteoglycans are synthesized by tumor and stromal cells and their biosynthesis is often dysregulated in malignancies, providing a favorable microenvironment for disease progression [2].

Serglycin is the only characterized intracellular proteoglycan till now and has been initially regarded as "hematopoietic" proteoglycan, being detected mainly in the secretory granules of hematopoietic cells [3, 4]. Numerous studies have shown that serglycin is constitutively secreted by tumor cells and in some cases is also located at the tumor cell membrane, although it does not hold a transmembrane domain [5–7]. Serglycin is highly expressed and secreted by tumor cells themselves and its overexpression is associated with tumor cell aggressiveness and poor disease outcome [8–10]. It is the major proteoglycan secreted by multiple myeloma cells affecting bone mineralization [7] growth of myeloma cell *in*

vivo and secretion of hepatocyte growth factor (HGF) [5]. Cell surface associated serglycin in myeloma cells is involved in cell adhesion to collagen type I and stromal cells [5, 11]. The adhesion of myeloma cells to collagen type I enhances the biosynthesis of matrix metalloproteinases (MMPs) [11]. Furthermore, secreted and cell surface associated serglycin is capable of inhibiting the classical and lectin pathways of complement via its chondroitin sulfate (CS) chains, thus protecting tumor cells from complement system attack [9, 12].

Few recent studies have demonstrated the overexpression of serglycin by aggressive cancer cells in tumors [8–10]. The upregulated biosynthesis and secretion of glycanated serglycin by cancer cells promote their growth, migration, and invasion and are correlated with poor prognosis [8–10]. Since little is known on the expression of serglycin in solid tumors, we went on to study the expression and distribution of serglycin in cancer cell lines and malignant tissues. In our pilot study, we show that serglycin is differentially expressed and secreted by breast, prostate, lung, and colon cancer cell lines. We identify the transcript variant of serglycin missing exon 2 in several of these cell lines. Our findings that serglycin is markedly synthesized by cancer and stromal cells in malignant tissues may propose a role for serglycin in cancer progression.

2. Materials and Methods

2.1. Antibodies, Enzymes, and Purified Proteins. Goat anti-rabbit horseradish peroxidase- (HRP-) conjugated secondary antibody was from Sigma-Aldrich. Rabbit polyclonal antibody against serglycin was prepared as previously described [7]. Chondroitinase ABC was purchased from Seikagaku. Serglycin isolated from culture medium of multiple myeloma cell lines was used as standard [7].

2.2. Cell Culture. All cell lines were purchased from the American Type Culture Collection (ATCC). MDA-MB-468, DLD-1, HT-29, A549, NCI-H23, NCI-H358, NCI-H661, HCC827, and PC-3 cells were cultured in RPMI 1640 medium (Biochrom) with 2 mM L-glutamine supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose (except for PC-3 cells) or 0.1 g/L (PC-3 cells), 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum as recommended by ATCC. MDA-MB-468 cells were also supplemented with 10 μg/mL human insulin (Sigma-Aldrich). MDA-MB-231, MCF-7, and CACO-2 cells were cultured in Eagle's minimum essential medium with Earle's BSS and 2 mM L-glutamine (EMEM, Biochrom) and supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 10 μg/mL human insulin (MCF-7 cells), and 10% fetal bovine serum (MDA-MB-231 and MCF-7 cells) or 20% fetal bovine serum (CACO-2 cells) as recommended by ATCC. For each cell line, 1% Pen/Strep (10000 units/mL penicillin and 10000 units/mL streptomycin, Biochrom) was used. Cells were cultured at 37°C in 5% CO_2.

2.3. Quantification of Serglycin Concentration in Culture Medium Supernatants. 1×10^6 cells were plated in 10 cm dish and cultured at normal culture conditions. After 18 h, cells were starved in serum-free medium for 48 h, when cultures reached 80% confluency and culture supernatants were collected. They were centrifuged at 3000 rpm for 5 min and were concentrated with Vivaspin 6 ultrafiltration devices (Sartorius Biotech). Protein concentration was measured by Coomassie Plus-Bradford Assay Kit (Thermo Scientific) and equal amounts of protein for every sample were treated with 0.02 units of chondroitinase ABC in 50 mM Tris-HCl pH 7.5 at 37°C for 2 h. Then, samples were reduced with β-mercaptoethanol in Laemmli sample buffer and were separated by SDS-PAGE electrophoresis. The proteins were transferred to Immobilon-P PVDF membranes (Millipore) and the membranes were blocked in 5% nonfat dry milk in PBS-0.1% Tween-20 for 2 h. Then, membranes were incubated with 0.55 μg/mL rabbit polyclonal anti-serglycin overnight at 4°C, washed three times with PBS-0.1% Tween-20, and then incubated for 1 h at room temperature with peroxidase-conjugated secondary goat anti-rabbit antibody. The immunoreactive proteins were detected by using the chemiluminescence horseradish peroxidase Pierce ECL western blotting substrate, according to the manufacturer's instructions. Serglycin content was analyzed through western blotting using increasing amounts of standard serglycin to create a standard curve each time [9]. Both culture supernatant and standard serglycin were treated with 0.02 units of chondroitinase ABC as above. The quantification of protein band density was performed using Scion Image software.

2.4. RNA Isolation and Real Time qPCR Analysis. 1×10^6 cells were plated in 10 cm dishes and cultured at normal culture conditions. After 18 h, cells were starved in serum-free culture medium for 48 h, when cultures reached 80% confluency. Total RNA was isolated from cells using NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany). The amount of isolated RNA was quantified by measuring its absorbance at 260 nm and the integrity of RNA was confirmed by electrophoresis on agarose gel stained with GelRed nucleic acid gel stain (Biotium). Total RNA was reverse transcribed using the PrimeScript 1st strand cDNA synthesis kit perfect real time (Takara Bio Inc., Japan) and KAPA Taq ReadyMix DNA Polymerase (KAPA BIOSYSTEMS). Real time PCR analysis was performed in 20 μL reaction mixture, according to the manufacturer's instructions using gene-specific primers (serglycin forward: 5′-GTTGGCGTGCAGCTGGGAGA-3′ and serglycin reverse: 5′-GGCTCTCCGCGTAGGATAACCTTG-3′, GAPDH forward: 5′-AGGCTGTTGTCATACTTCTCAT-3′ and GAPDH reverse: 5′-GGAGTCCACTGGCGTCTT-3′). The amplification was performed using Rotor Gene Q (Qiagen, USA). All reactions were performed in triplicate and a standard curve was always included for each pair of primers for assay validation. In addition, a melting curve analysis was always performed for detecting the SYBR Green-based objective amplicon. To provide quantification, the point of product accumulation in the early logarithmic phase of the amplification plot was defined by assigning a fluorescence threshold above the background, defined as the threshold cycle (Ct) number. Relative expression was calculated by the ΔΔCt

method. The Ct of any gene of interest was normalized to the Ct of the normalizer (GAPDH).

2.5. DNA Fragment Identification. In order to isolate and sequence PCR products for serglycin gene expression within the cell lines reverse transcription-polymerase chain reaction (RT-PCR) was performed with DyNAzyme II DNA Polymerase kit (Finnzymes). Serglycin and β-actin transcript levels were detected using gene-specific primers (serglycin forward: 5′-AATGCAGTCGGCTTGTCCTG-3′ and serglycin reverse: 5′-TGGTGTCAAGGTGGGAAAAT-3′ resulting in amplicon size of 483 bp for full-length serglycin and 336 bp for serglycin transcript variant lacking exon 2, β-actin forward: 5′-GTGGGGCGCCCCAGGCACCA-3′ and β-actin reverse: 5′-CTCCTTAATGTCACGCACGATTTC-3′ resulting in amplicon size of 539 bp). For PCR reaction, samples containing 125 ng of cDNA were amplified in a total volume of 50 μL [10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, containing dNTP mix (each at 0.2 mM), both downstream and upstream primers (each at 200 nM), and 1 unit of DyNAzyme II DNA polymerase]. The PCR amplification was carried out as follows: 95°C for 20 sec, annealing temperature 51°C for 20 sec, and 72°C for 30 sec (MiniCycler, MJ Research). Equal volumes of the PCR products were electrophoresed on 1% agarose gel stained with GelRed nucleic acid gel stain (Biotium). For sequencing experiments, PCR products for serglycin were isolated from MDA-MB-231 and A549 cancer cells. The bands correspond to full-length serglycin and serglycin transcript variant lacking exon 2 was cut from the agarose gel and the DNA fragments were extracted and purified using Nucleospin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions. DNA fragments were sequenced by automated sequencing in the institutional facility at the Karolinska Institute (Stockholm, Sweden).

2.6. Tissue Samples and Immunohistochemistry. In the present study, we used a tissue microarray (TMA) platform that contained 10 cases of each of the following 4 types of carcinomas: colon, breast, lung, and prostate (TP483 for colon, breast, prostate, and lung cancer, US Biomax Inc.). Clinicopathological data, including gender, age, tumor grade, and TNM stage, were available. Following deparaffinization in xylene and rehydration in graded ethanol, conventional immunohistochemistry was performed on the TMA slide as described previously [9], with the use of rabbit anti-serglycin antibody (1.38 μg/mL). Specific binding was detected with the Dako REAL EnVision detection system (peroxidase/DAB+, rabbit/mouse) and visualized with diaminobenzidine. Myeloma section was used as a positive control. In negative control slides, the primary antibody was substituted with 1% TBS. The sections were counterstained with hematoxylin and serglycin staining was scored as follows: 0, no staining; 1+, weak; 2+, moderate; 3+, strong staining.

3. Results

3.1. Serglycin Is Differentially Expressed by Cancer Cells. Total RNA from A549, NCI-H23, NCI-H358, NCI-H661, and HCC827 lung cancer cells; MDA-MB-231, MDA-MB-468, and MCF-7 breast cancer cells; PC-3 prostate cancer cells; and CACO-2, DLD-1, and HT-29 colon cancer cells cultured in serum-free medium was reverse transcribed and amplified using specific primers for serglycin (Figure 1(a)). It was found that the expression of serglycin differs among cell lines and is likely aggressive-specific (Figure 1(a)). We have previously investigated serglycin expression in breast cancer cell lines MDA-MB-231, MDA-MB-468, and MCF-7 [9] of different aggressiveness [13, 14] by reverse transcription PCR. We went on to analyze the expression of serglycin in these breast cancer cell lines by qPCR and compare it to serglycin expression in cancer cell lines of different origin and tumorigenicity. In agreement with data presented in our previous study [9], serglycin was expressed in minute levels in low aggressive MDA-MB-468 and MCF-7 cells, whereas it was expressed in elevated levels in high aggressive MDA-MD-231 breast cancer cells, which are $KRAS^{38G\to A}$ and $BRAF^{1391G\to T}$ mutant (Figure 1(a)) [13, 14]. In accordance with these data, highly tumorigenic $KRAS^{12G\to S}$ mutation activated A549 [15, 16] and $HER1/EGFR^{\Delta E746-750}$ kinase domain mutation activated HCC827 lung cancer cells [17] expressed much higher levels of serglycin as compared to less tumorigenic $KRAS^{12G\to C}$ mutant NCI-H358 and NCI-H23 as well as nonmutated H661 lung cancer cells [15, 16, 18] (Figure 1(a)). Serglycin expression was also significantly elevated in highly aggressive and tumorigenic [19] PC-3 prostate cancer cells (Figure 1(a)). The expression of serglycin was found to be relatively low in colon cancer cell lines and surprisingly was higher in low aggressive CACO-2 cells as compared to more aggressive HT-29 (mutant $BRAF^{600V\to T}$) and DLD-1 (mutant $KRAS^{13G\to D}$) colon cancer cells [20, 21] (Figure 1(a)).

In order to evaluate whether serglycin is secreted in the culture medium of cancer cells, they were cultured in serum-free medium and supernatants were collected and concentrated. Equal amounts of protein for every sample were digested with chondroitinase ABC and analyzed by western blot (Figure 1(b)). Serglycin core protein was detected only in the culture medium of aggressive cell lines such as A549, NCI-H23, and HCC827 lung cancer cells and DLD-1 colon cancer cells as well as MDA-MB-231 breast cancer cells as previously shown [9], which harbor KRAS or HER1/EGFR mutations (Figure 1(b)). It is worth noticing that aggressive NCI-H23 and DLD-1 cancer cells expressing low mRNA levels for serglycin secreted significant amounts of this proteoglycan. In contrast, aggressive PC-3 cells that highly express mRNA for serglycin did not secrete detectable amounts of this proteoglycan. We did not analyze cell culture supernatants from NCI-H358 lung cancer cell which are also KRAS mutant. We went on to quantify the secreted serglycin by western blot analysis creating a standard curve using various amounts of standard serglycin. Using standard curves, we simultaneously analyzed serglycin present in the culture medium of cancer cells and we found that the concentration of serglycin was as follows: A549, 0.86 ± 0.07 μg/mL; NCI-H23, 0.43 ± 0.02 μg/mL; HCC827, 0.82 ± 0.12 μg/mL; MDA-MB-231, 0.6 ± 0.16 μg/mL; and DLD-1, 0.14 ± 0.05 μg/mL (Figure 1(c)).

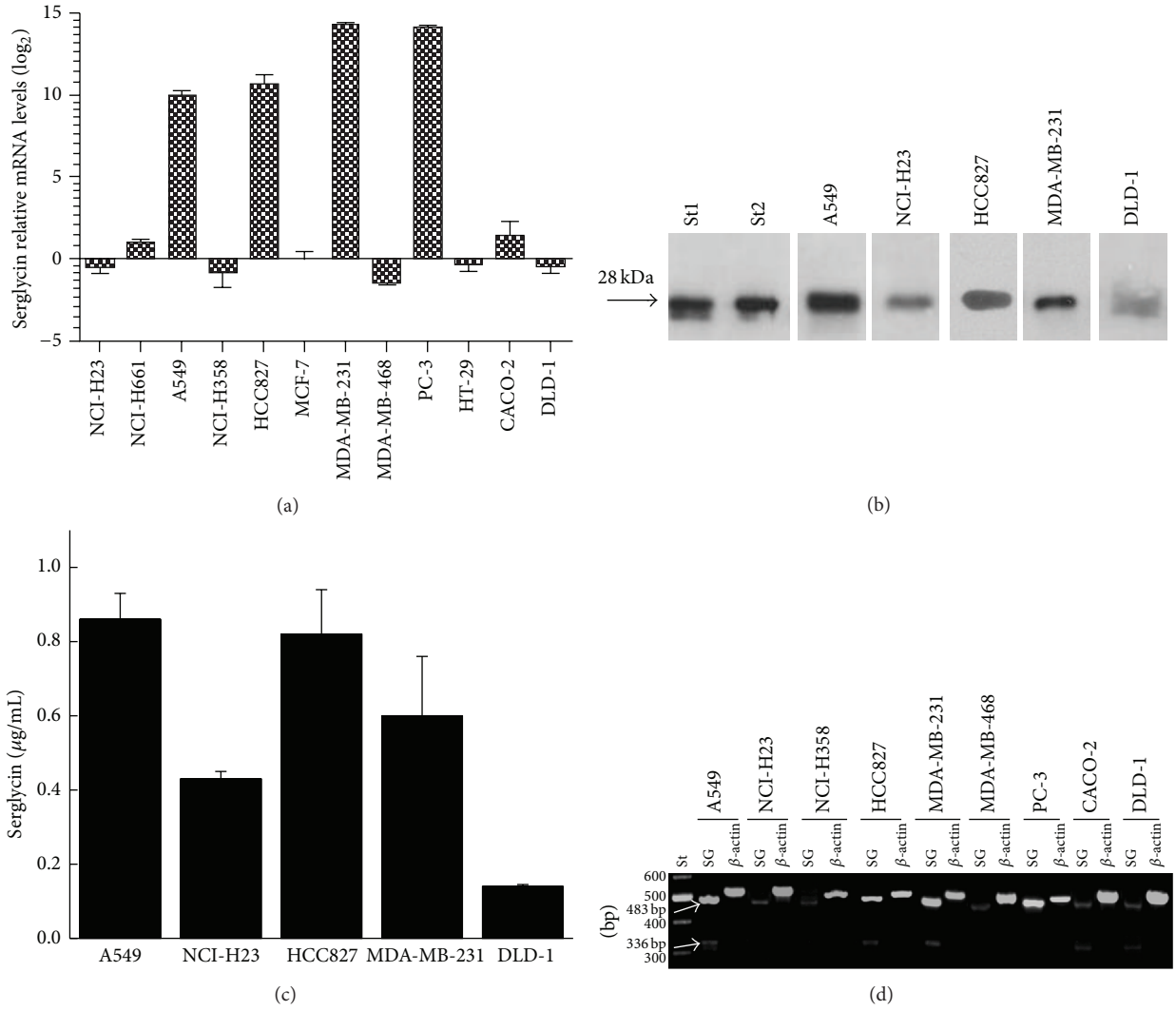

Figure 1: (a) Expression of serglycin across twelve cancer cell lines of lung (NCI-H23, NCI-H661, A549, and NCI-H358), breast (MCF-7, MDA-MB-231, and MDA-MB-468), prostate (PC-3), and colon (HT-29, CACO-2, and DLD-1). Results are mean of three separate experiments performed in triplicate ± S.D. (b) Equal amounts of protein from concentrated cell culture supernatants of cancer cell lines were treated with chondroitinase ABC and subjected to western blot analysis for serglycin using chondroitinase ABC digested standard serglycin (st1 and st2) as positive control. (c) Quantification of serglycin secreted in cell culture supernatants. Results are mean of three separate experiments performed in triplicate ± S.D. (d) Detection of serglycin gene transcripts by RT-PCR. The PCR products were analyzed on 1% agarose gels stained with GelRed.

3.2. Identification of Alternative Splicing of Serglycin in Cancer Cells. RT-PCR followed by agarose electrophoresis revealed the presence of two bands of RT-PCR products in A549 and HCC827 lung cancer cells; MBA-MB-231 breast cancer cells; and CACO-2 and DLD-1 colon cancer cells (Figure 1(d)). The upper band was the expected 483 bp fragment that represents the full-length sequence between exons 1 and 3 (bases 106–589) of human serglycin gene (Figure 1(d)). The lower band of 336 bp might correspond to an exon 2 deletion (Figure 1(d)). To identify both bands, RNA isolated from MDA-MB-231 and A549 cancer cells was subjected to RT-PCR followed by agarose electrophoresis. Both bands were extracted from the gel, purified, and sequenced. The sequencing verified the 483 bp fragment as the product of full-length sequence between exons 1 and 3 (bases 106–589) of human serglycin gene and showed that the reduced size of the 336 bp band was due to complete absence of exon 2 of serglycin gene. The partial sequence around this alternative splice site was tggaatcctcagttcaaGAagacgagaatccaggac, where the capital letters show the junction between exon 1 and exon 3 of serglycin gene. The loss of exon 2 (49 amino acids) has been also demonstrated in human neutrophils [22]; however, alternative splicing of serglycin in tumor cells has not been previously reported.

3.3. Distribution of Serglycin in Normal and Malignant Tissues

3.3.1. Colon Cancer. In the present tissue microarray, there were 10 colon carcinomas of different grades (well differentiated $n = 2$, moderately differentiated $n = 6$, and

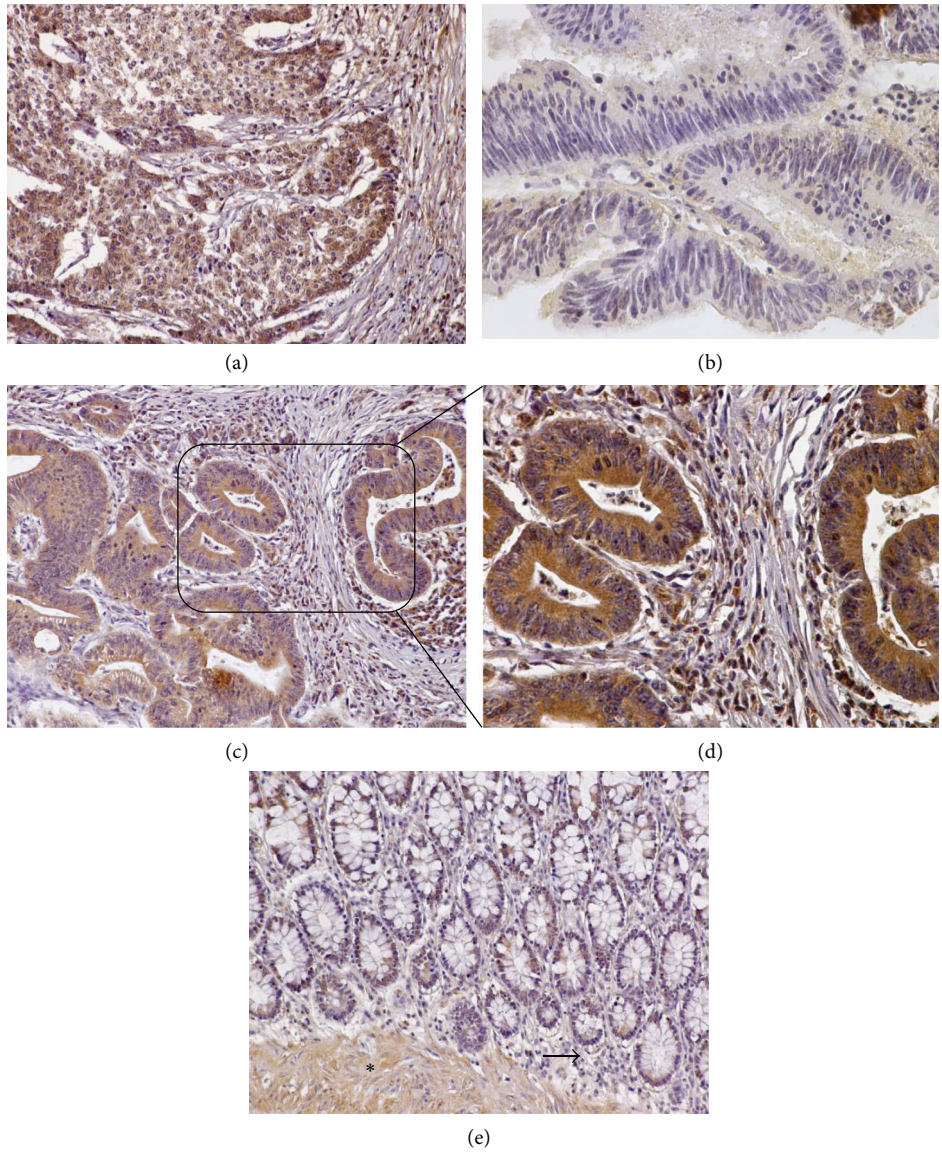

Figure 2: (a) Intense, diffuse cytoplasmic serglycin staining in a poorly differentiated colon adenocarcinoma. (b) Weak immunopositivity for serglycin in a well-differentiated adenocarcinoma, arising from an adenoma. (c) Moderately differentiated colon adenocarcinoma displaying strong immunoreactivity for serglycin. (d) Lager magnification showing that plasma cells, lymphocytes, and stromal cells of the invasive front are positive for serglycin. (e) Normal colon glands exhibit moderate cytoplasmic levels for serglycin. Note that muscularis mucosa (asterisk) is positive for serglycin, whereas staining is weak in plasma cells, lymphocytes, and stromal cells of the lamina propria (arrow). (a, b, c, e) Original magnification 20x; (d) original magnification 40x.

poorly differentiated $n = 2$) and normal colonic epithelia ($n = 2$). The expression of serglycin was diffuse, granular, and almost exclusively cytoplasmic in all the colon cancer cases, as well as in normal colon epithelia (Figure 2). Notably, grade 2 and 3 neoplasms displayed very strong serglycin immunoreactivity (Figures 2(a), 2(c) and 2(d)), whereas the intensity of the two grade 1 malignancies was weak (+1) and moderate (+2) (Figure 2(b)). The case that exhibited weak immunopositivity was a low-grade adenocarcinoma, originating from a villous adenoma (Figure 2(b)). A careful examination of the samples revealed that, in all malignancies lymphocytes, plasma cells, stromal and endothelial cells were immunopositive for serglycin (Figure 2(d)). Interestingly, the intensity of serglycin immunoexpression was augmented at the invasive front of the carcinomas (Figure 2(d)). Normal large intestine epithelium displayed moderate serglycin immunoreactivity, whereas serglycin staining was weak in plasma cells, lymphocytes, and stromal cells of the lamina propria (Figure 2(e)). Smooth muscle cell layer of colon also displayed elevated serglycin immunoreactivity (Figure 2(e)).

3.3.2. Breast Cancer. The tissue microarray also contained 7 grade 2 and 3 grade 3 breast carcinomas, as well as 2 cores obtained from 2 different normal breasts. All the neoplasms

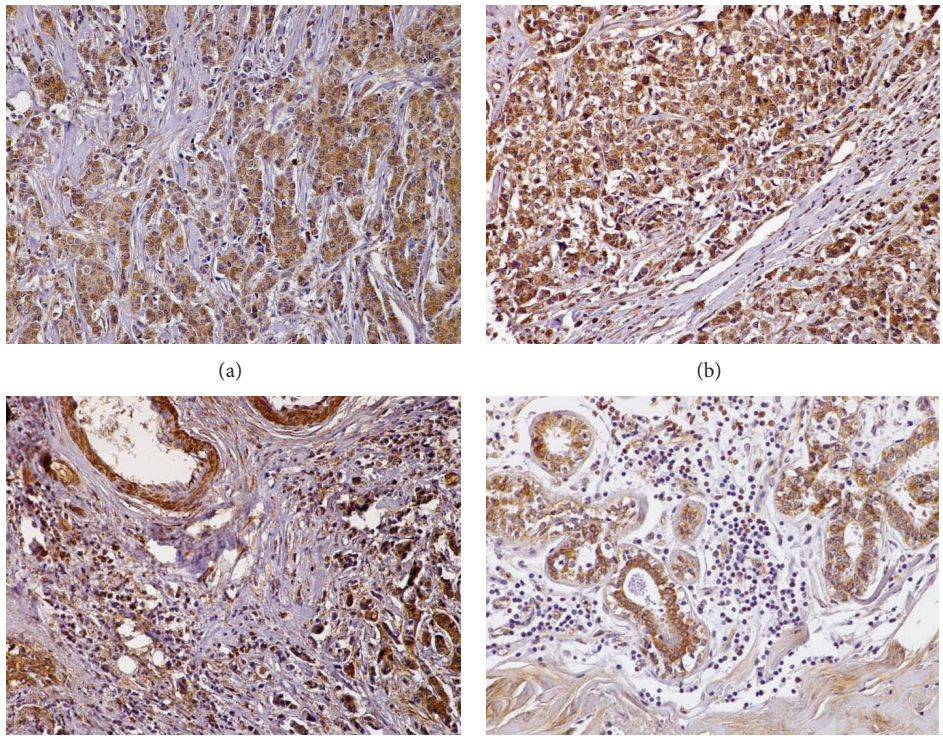

Figure 3: (a) Grade 2 and (b) grade 3 breast ductal carcinomas displaying strong granular, cytoplasmic serglycin immunoreactivity. (c) Grade 3 infiltrating breast carcinoma. Note that the tumor cells as well as chronic inflammatory and stroma cells show strong serglycin expression. (d) Section from a benign breast lesion showing that serglycin is expressed in the glands and the stroma. However, inflammatory cells display minimal serglycin positivity. Original magnifications 20x.

and the normal glands displayed diffuse, strong (3+), cytoplasmic, granular serglycin immunoreactivity (Figures 3(a)–3(d)). The vast majority of plasma cells, lymphocytes, and endothelial and stromal cells were also serglycin positive. Of note, the expression of serglycin levels was enhanced in the plasma cells, lymphocytes, and stromal cells from grade 3 tumors (Figure 3(c)). The expression of serglycin in the lymphocytes and plasma cells of normal tissues was minimal (Figure 3(d)).

3.3.3. Prostate Cancer. Two low-grade (Gleason score 4), 3 moderate-grade (Gleason score 6), and 5 high-grade prostate adenocarcinomas (Gleason score 10) were examined for serglycin expression. Serglycin was detected in both the neoplastic and the normal prostatic epithelia. Its immunoexpression was cytoplasmic, granular, and diffuse in all the tumors examined (Figures 4(a)–4(d)). Interestingly, none of the low-/moderate-grade tumors displayed high (3+) serglycin cellular levels. On the contrary, there was no high-grade adenocarcinoma exhibiting low-grade (1+) serglycin immunoreactivity in the samples tested. Endothelial cells in tumor stroma had elevated serglycin cellular levels (Figure 4(c)). Low levels of serglycin were also detected in the cytoplasm of hyperplastic and normal prostate glands (Figures 4(e) and 4(f)). Notably, basal cells of prostatic glands and fibrovascular cores were negative (Figure 4(f)), while smooth muscle and corpora amylacea were positive for this proteoglycan (Figures 4(e) and 4(f)).

3.3.4. Lung Cancer. We also examined the distribution of serglycin in 5 squamous cell carcinomas (moderately and poorly differentiated) and 5 adenocarcinomas (moderately and poorly differentiated), including a large cell carcinoma and a bronchoalveolar carcinoma. The intensity of serglycin immunoreactivity was strong (+3) and its distribution was diffuse, cytoplasmic, and granular in all the lung carcinomas examined (Figures 5(a)–5(d)). Endothelial cells, plasma cells, and lymphocytes displayed intense serglycin immunoreactivity. Stromal fibroblasts were also serglycin positive, primarily at the invasive fronts (Figure 5(a)). Notably, in the one case of large cell carcinoma that was involved in the tissue microarray, the tumor cells displayed strong membrane immunoreactivity as well as nuclear staining (Figure 5(d)). As regards normal lung, serglycin was strongly expressed in macrophages, pneumocytes, and bronchial epithelium cells (Figures 5(e) and 5(f)).

4. Discussion

Although numerous studies have shown that serglycin is involved in hematological malignancies, not much information has been published on the expression and distribution of

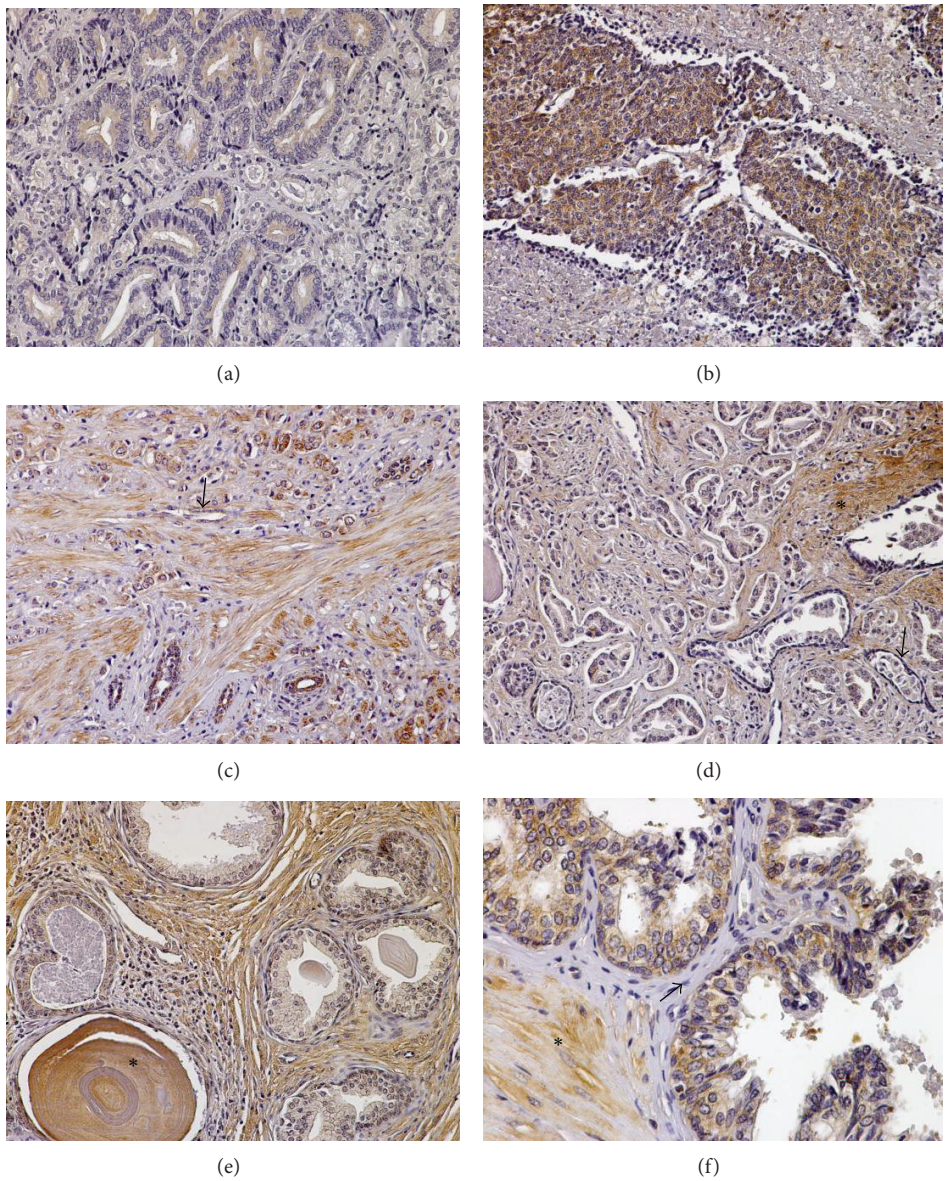

Figure 4: (a) A low-grade prostate adenocarcinoma showing weak serglycin expression. (b) A high-grade prostate adenocarcinoma with strong cytoplasmic, diffuse immunoreactivity for serglycin. (c) Note that tumor, endothelial (arrow), and stroma cells are positive for serglycin in this high-grade prostate adenocarcinoma. (d) In this case of low-grade prostatic adenocarcinoma, malignant and benign prostate glands, as well as fibromuscular stroma (asterisk), display serglycin immunopositivity; on the contrary, basal cells of the benign prostate glands (arrow) are negative for this protein. (e) Benign prostate hyperplasia. Benign glands, stromal cells, chronic inflammation cells (plasma cells and lymphocytes), and corpora amylacea (asterisk) are serglycin-reactive. Also note that basal cells do not exhibit serglycin immunoreactivity. (f) In this section of normal prostate, epithelial cells and smooth muscle (asterisk) are positive, whereas fibrovascular core cells (arrow) are negative for serglycin. (a–e) Original magnification 20x; (f) original magnification 40x.

this proteoglycan in solid tumors [1, 2, 23]. Only few studies have shown that serglycin augmented expression is associated with cancer cell aggressiveness and disease progression in hepatocellular [8], breast [9], and nasopharyngeal cancer [10].

The results of the present pilot study reveal that serglycin is highly expressed and secreted by more aggressive cancer cells. Serglycin is secreted in elevated levels in cancer cells mutated in KRAS or HER1/EGFR, the fact that may suggest an implication of EGFR-RAS pathway in the biosynthesis and more importantly in secretion of serglycin. As shown in previous studies, the overexpression of serglycin is correlated with the establishment of more aggressive mesenchymal cancer cell phenotype and promotes cancer cells proliferation, especially in nonadhesive matrices, migration, and invasion both *in vitro* and *in vivo* [9, 10]. All cancer cells analyzed till now synthesize serglycin, which is modified with CS chains and not heparin as in mast cells [3, 4]. The glycanation of serglycin may modulate the biological functions of tumor-derived serglycin since it seems to be important for serglycin's biological functions [23]. For example, serglycin

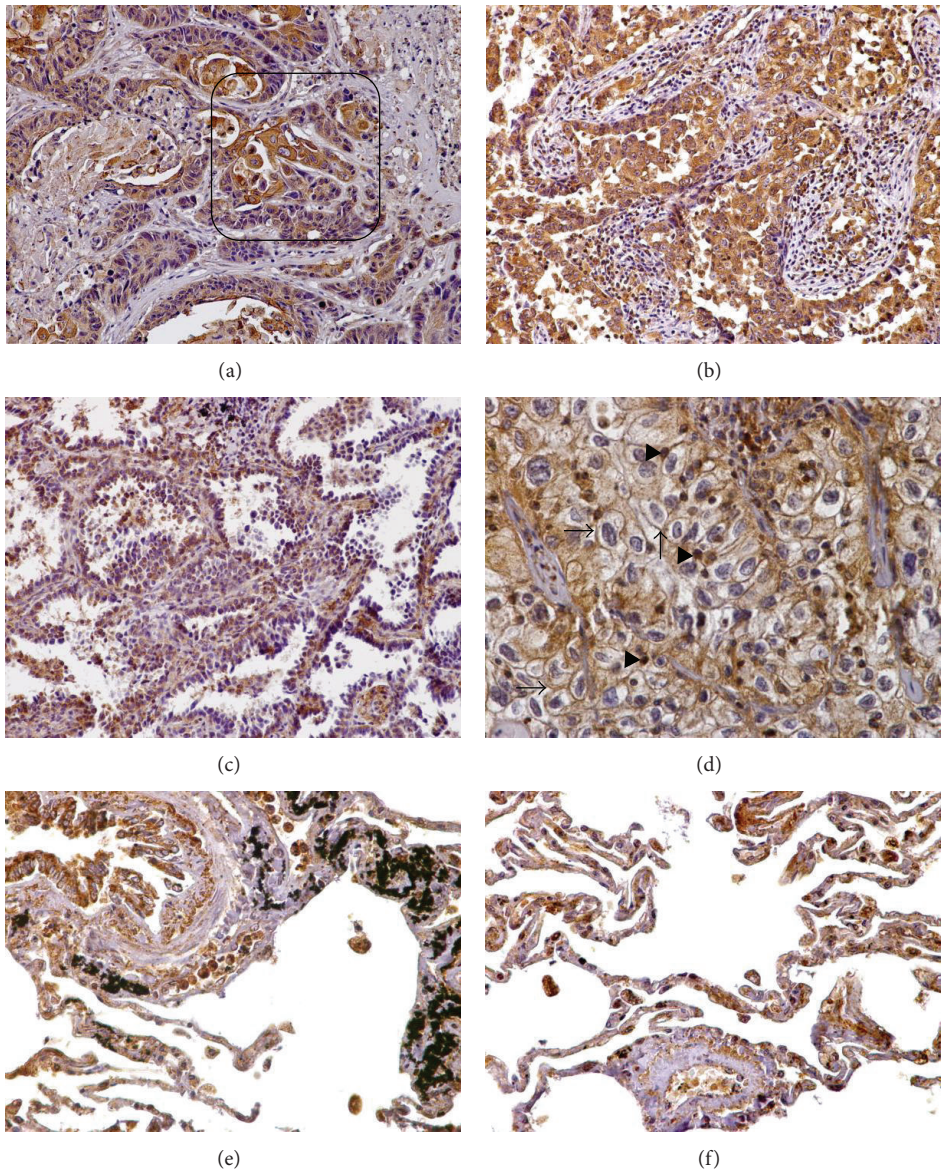

FIGURE 5: (a) A moderately differentiated squamous cell lung carcinoma displaying strong cytoplasmic serglycin immunoreactivity. Note that stroma and chronic inflammation cells are also intensively positive for serglycin, primarily at the invasive fronts (box). (b) A moderately differentiated lung adenocarcinoma that shows strong serglycin immunoexpression. (c) Serglycin immunopositivity in a low-grade bronchoalveolar adenocarcinoma. (d) Note that in this case of large cell undifferentiated lung carcinoma serglycin mainly displays cytoplasmic, granular immunoreactivity; however, it also displays nuclear (arrowheads) and the cell membranous (arrows) localization. (e and f) In normal lung sections, serglycin is detected in the epithelium of bronchioles as well as in alveolar macrophages and pneumocytes. Original magnifications 20x.

modified with CS chains enriched with 4-sulfated disaccharides is responsible for conferring tumor cells resistance against immune system attack mediated by the activation of the classical and lectin pathways of complement [9, 12]. Furthermore, the establishment of aggressive breast cancer phenotype following upregulation of NEDD9 is accompanied by increased expression of serglycin, its cell surface binding partner CD44, and CS synthesizing enzymes [24]. This study has demonstrated that serglycin and CD44 carry CS modified with disulfated disaccharides being sulfated at C4 and C6 of *N*-acetyl-galactosamine (CS-E units). It has been shown that CS-E plays a key role in promoting and regulating breast cancer progression and metastasis and possibly stem cell phenotype [24]. CS chains of serglycin are mainly responsible for binding to CD44 and collagen type I. Serglycin binding to CD44 most likely mediates the cell surface localization of serglycin [5, 7] that further interacts with matrix collagen type I thus promoting the adhesion of tumor cells [5, 11]. The core protein of serglycin is involved in the interaction with MMP-13 [25] and proMMP-9 and the formation of complexes [26, 27]. Both hemopexin-like domain and the fibronectin-like module of proMMP-9 are implicated in the interaction

with serglycin. The formation of complexes modulates the mode of activation of the enzyme and its interaction with substrates [26, 28].

In our study, the expression of a splice variant of serglycin that lacks exon 2 was detected although the mature protein encoded by this splice variant was not detected in our western blots likely due to its minor expression. The alternative splicing that occurs from the loss of exon 2 has been previously demonstrated in neutrophils and has been associated with the presence of a unique DNase I-hypersensitive site in exon 2 in these cells [22]. The biological importance of exon 2 deletion for serglycin is unclear. Nevertheless, it may participate in several serglycin functions since the N-terminal portion of the sequence encoded by exon 2 contains a potential heparin binding site YPTQRARYQWVRCNP and the possible dimerization of the peptide sequence via the cysteine residue results in significant binding affinity to low molecular weight heparin [29]. It has been proposed that heparin binding site and dimerization of serglycin core together with glycosaminoglycan chains regulate the binding of serglycin with other molecules [23, 29].

The biosynthesis of serglycin by normal and cancer cells themselves was detected in cancer tissues by immunohistochemistry. Serglycin was found to be expressed in normal epithelial cells exhibiting cytoplasmic staining. The biological function of serglycin in normal epithelium is still unknown. Further studies are needed to elucidate whether serglycin is located within vesicles in epithelial cells, is either secreted constitutively or upon stimulation, or remains exclusively intracellularly. Although a limited number of cases were examined, our data have shown a strong diffuse cytoplasmic distribution for serglycin in more aggressive cancer cells in most cases. The pattern of cytoplasmic staining was granular suggesting the packaging of serglycin in secretory granules within cancer cells. It is known that serglycin is localized in secretory granules in hematopoietic cells as well, where it plays a role in the packaging and secretion of many molecules [23]. A similar function of serglycin in cancer cells cannot be excluded.

In this pilot study, serglycin was also found to be expressed in higher levels in stromal cells in tumor stroma as compared to normal tissue. Serglycin was expressed by inflammatory and endothelial cells and fibroblasts in reactive tumor stroma especially at the invasive fronts suggesting a crucial role for serglycin in cancer spread. It has been shown that serglycin expression is markedly upregulated in cancer-activated fibroblasts among other inflammatory mediators and a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS-1) promoting cancer cell invasion [30]. Serglycin is also implicated in the biosynthesis and secretion of growth factors and inflammatory mediators such as HGF and CXCL1 in plasma [5] and endothelial cells [31, 32], respectively, as well as in the production of proteases in several inflammatory cells, which have proven roles in cancer cell growth and spread [3, 23]. Recently, it has been shown that serglycin is highly expressed in tumors in RIP1-Tag2 mouse model for spontaneous insulinoma formation [33]. It is suggested that the majority of serglycin comes from stromal cells. When RIP1-Tag2 mouse model is crossed into serglycin deficient mice, a decrease only in the volume of developed tumors but not in their number is noticed [33]. Furthermore, the frequency of angiogenic islets is decreased in serglycin deficient RIP1-Tag2 mice and this is also accompanied with reduced biosynthesis of proangiogenic modulators such as vascular endothelial growth factor and HGF [33]. The absence of serglycin also enhances the functionality of tumor vessels, which were better perfused than that developed in tumors in serglycin wild type mice [33]. These findings suggest the involvement of serglycin in the development of a proangiogenic environment.

The overexpression of serglycin by cancer and stromal cells may augment the expression of inflammatory mediators, growth factors, and proteolytic enzymes. These factors may act in autocrine and/or paracrine manner affecting the behavior of both stromal and cancer cells. It is likely that serglycin is implicated in the establishment of a flourishing inflammatory tumor microenvironment that drives cancer cell growth and spreading.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Organotypic Cultures of Intervertebral Disc Cells: Responses to Growth Factors and Signaling Pathways Involved

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Intervertebral disc (IVD) degeneration is strongly associated with low back pain, a major cause of disability worldwide. An in-depth understanding of IVD cell physiology is required for the design of novel regenerative therapies. Accordingly, aim of this work was the study of IVD cell responses to mitogenic growth factors in a three-dimensional (3D) organotypic milieu, comprising characteristic molecules of IVD's extracellular matrix. In particular, annulus fibrosus (AF) cells were cultured inside collagen type-I gels, while nucleus pulposus (NP) cells in chondroitin sulfate A (CSA) supplemented collagen gels, and the effects of Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), and Insulin-Like Growth Factor-I (IGF-I) were assessed. All three growth factors stimulated DNA synthesis in both AF and NP 3D cell cultures, with potencies similar to those observed previously in monolayers. CSA supplementation inhibited basal DNA synthesis rates, without affecting the response to growth factors. ERK and Akt were found to be phosphorylated following growth factor stimulation. Blockade of these two signaling pathways using pharmacologic inhibitors significantly, though not completely, inhibited growth factor-induced DNA synthesis. The proposed culture systems may prove useful for further in vitro studies aiming at future interventions for IVD regeneration.

1. Introduction

Low back pain has been reported to be the leading cause of disability worldwide [1] having a great impact on the health care system and society [2]. It is strongly associated with intervertebral disc (IVD) degeneration [3]. IVDs lie between the vertebral bodies of the spinal column providing mechanical support and flexibility to the body and absorbing the loads and vibrations that result from the standing position and the specific activities of each person [3]. IVDs consist of an outer layer of laminated fibres (containing fibroblast-like cells) and a gelatinous core (with cells resembling chondrocytes) called annulus fibrosus (AF) and nucleus pulposus (NP), respectively [3]. AF is characterized by a well-organized network of concentric collagen lamellae, with collagen type-I being the predominant extracellular matrix (ECM) constituent [4]. On the other hand, NP mostly comprises collagen type-II and proteoglycans, especially aggrecan, which maintains tissue hydration due to its chondroitin and keratan sulfate chains [3].

IVD degeneration is characterized by tissue disorganization and vascular and neural infiltration, a fact associated with the discogenic back pain [5]. Changes at the molecular and biochemical levels have been observed in the degenerated IVDs, such as loss of proteoglycans and water [6] and increased expression of matrix metalloproteinases and aggrecanase [7]. Many of these changes have been associated with alterations in the expression levels of various growth factors and their receptors [8, 9]. Currently, IVD degeneration is mainly treated with medication aiming at pain relief or—in more severe cases—with surgical interventions, such as discectomy, spinal fusion, or disc replacement, all of which however exhibit many clinical contraindications and possible catastrophic complications [10]. Hence, novel therapies aiming at the regeneration of the degenerated disc have been suggested, such as cell transplantation [11, 12] or growth factor injections [13, 14]. Nevertheless, for the successful outcome of such efforts, the in-depth understanding of disc cell physiology is necessary, especially regarding the proliferative responses to growth factors. We have previously

reported that Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), and Insulin-Like Growth Factor-I (IGF-I) stimulate the proliferation of bovine IVD cells in vitro via the activation of the ERK and Akt signaling pathways [15]. Furthermore, we have shown that the same growth factors added in human IVD cells, as well as autocrine factors produced by them, stimulate their proliferation via the same two signaling pathways [16]. These previous studies have been conducted using the conventional monolayer cell culture approach, which does not approximate very well the in vivo environment of the tissue. Accordingly, aim of the present report was the examination of bovine IVD cell proliferative responses to these three growth factors using three-dimensional (3D) culture systems. In an effort to simulate the cells' in vivo environment, proteins encountered in abundance in the two IVD compartments were used; that is, AF cells were cultured inside collagen type-I gels, while NP cells were cultured in collagen gels supplemented with chondroitin sulfate A (CSA).

2. Materials and Methods

2.1. Materials. Human recombinant (h.r.) PDGF-BB (the PDGF-isoform considered to represent the universal ligand for all PDGF receptor subtypes [17]), h.r. bFGF, and h.r. IGF-I were purchased from R&D Systems (Minneapolis, MN, USA). Chondroitin sulfate A sodium salt from bovine trachea (CSA), PD98059, wortmannin, LY294002, calphostin C, Y-27632 dihydrochloride, protease and phosphatase inhibitor cocktails, 5-bromo-2′-deoxyuridine (BrdU), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma (St. Louis, MO, USA). The rabbit anti-phospho-Akt (Ser473) and anti-Akt1/2/3 antibodies were obtained from Cell Signaling Technology (Hertfordshire, UK), while mouse anti-phospho-ERK1/2 antibody that recognizes phosphorylated Thr202/Tyr204 and mouse anti-pan-ERK antibodies were obtained from BD Transduction Laboratories (Bedford, MA, USA). The FITC-conjugated anti-BrdU monoclonal antibody (clone BMC9318) was from Roche Diagnostics GmbH (Mannheim, Germany). [Methyl-^{3}H]-thymidine was from Moravek Biochemicals (Brea, CA, USA). Crude collagenase, cell culture media, antibiotics, and sodium pyruvate were purchased from Biochrom KG (Berlin, Germany), except for the low glucose (1,000 mg/L) formulation of Dulbecco's minimal essential medium (DMEM), trypsin, and fetal bovine serum (FBS) which were from Gibco, Life Technologies Europe BV (Thessaloniki, Greece).

2.2. Cell Isolation and Cell Culture Conditions. Tails from young steers (8–12 months of age) were obtained from a local slaughterhouse and they were processed within 8 h after slaughter, as described [15]. Briefly, nucleus pulposus (NP) and outer annulus fibrosus (AF) were isolated based on visual inspection, and each part was further minced in small pieces (approximately 1 mm^3), which were subjected to an overnight digestion with a crude collagenase solution in DMEM (1 mg/mL for NP and 3 mg/mL for AF). Cells were recovered by centrifugation and they were routinely cultured in DMEM (high glucose formulation, i.e., 4,000 mg/L) supplemented with penicillin and streptomycin, sodium pyruvate, L-glutamine, and 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C. Cells were routinely subcultured when confluent by using a trypsin/citrate (0.25/0.30% w/v) solution. Cell counting, after trypsinization, was performed by using a Coulter counter (Beckman-Coulter, Fullerton, CA). Cells were tested periodically and found to be mycoplasma-free.

2.3. Preparation of Three-Dimensional Cell Cultures. Collagen was extracted from rat-tail tendons according to a modification of the method of Bell et al. [18]. Briefly, tendons were solubilized under aseptic conditions in 0.1% (v/v) acetic acid, for 48 h, at 4°C. The solution was centrifuged at 10,000 rpm in a Sorvall (DuPont) model RC-5C centrifuge in an SS-34 rotor for 90 min and the supernatant was stored at 4°C. This stock solution contained approximately 4 mg/mL total protein and consisted primarily of collagen type-I. Alternatively, a commercially available rat-tail collagen type-I solution was used (BD Biosciences, Bedford, MA, USA) containing 4.01 mg/mL total protein in 0.02 N acetic acid. Equivalent results were obtained using both collagen type-I solutions.

In order to form 3D cell-populated collagen gels, a premix of the collagen solution with DMEM 10x and $NaHCO_3$ 7.5% at a ratio of 17 : 2 : 1 was prepared. This was used for AF cells or it was enriched with CSA at the indicated concentrations to be used for NP cells. Cell pellets (10^6 cells/mL of solution) and FBS (at a final concentration of 0.1%) were added to the premix, mixed further to ensure a homogeneous distribution of the cells and layered on the culture dishes. After 30 min at 37°C for polymerization, low glucose formulation of DMEM containing 0.1% FBS was layered on top of the gels.

2.4. Tritiated Thymidine Incorporation Assay. 3D cell cultures were left for 2 days in low glucose formulation of DMEM containing 0.1% FBS. Then fresh medium was added along with the growth factors to be tested and methyl-[^{3}H]-thymidine (0.2 μCi/mL, 25 Ci/mmol). After 24 h of incubation, the medium was aspirated and the collagen gels were digested for 1 h at 37°C, with a crude collagenase solution (1 mg/mL in 130 mM NaCl, 10 mM $CaCl_2$, 10 mM HEPES, pH 7.2). The cells were collected by centrifugation (1000 ×g, 5 min) and lysed in 0.3 N NaOH/1% SDS solution for 1 h. Ice-cold TCA (f.c. 10%) was added to the lysates, which were kept at 4°C for 1 h more. The lysates were then filtered through GF/B glass-fiber filters (Sigma). The filters were air-dried and subjected to scintillation counting, as previously described [19].

When indicated, the cells were preincubated with the appropriate concentrations of kinase inhibitors for 45 min before growth factor treatment.

2.5. Bromodeoxyuridine Incorporation Assay. IVD cells were plated overnight on glass coverslips at a density of 2×10^4 cells/cm^2, in DMEM containing 10% FBS. They were growth-arrested for 48 h in low glucose formulation of DMEM containing 0.1% FBS and then stimulated for 24 h with PDGF-BB (10 ng/mL) in medium supplemented with

50 μM BrdU, in the presence or absence of CSA (250 μg/mL). The cells were fixed in freshly prepared solution of 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, treated with 2 N HCl, and incubated with FITC-conjugated anti-BrdU-mAb (at 4°C, overnight) followed by staining with 1 mg/mL DAPI in PBS (10 minutes) in the dark at room temperature. Cells were washed 3 times with PBS at each step. DAPI- and BrdU-positive nuclei were observed on a Zeiss Axioplan 2 fluorescence microscope with a 40x objective; a field containing approximately 200 cells was used for quantification purposes. Images were captured using a ProgRes CF cool CCD camera (Jenoptik Optical Systems GmbH, Jena, Germany) controlled by a PC equipped with the ProgRes CapturePro software (Jenoptik).

2.6. Western Analysis. 3D cell cultures were left for 2 days in low glucose formulation of DMEM containing 0.1% FBS. Then fresh medium was added along with the growth factors to be tested for the indicated time periods. For the collection of cell lysates, 3D gels were washed with ice-cold Tris buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 15 mM NaCl), carefully detached from the culture dishes and transferred to Eppendorf tubes. After a brief centrifugation (10,000 ×g, 3 min, 4°C), the supernatant was discarded, and gels including the cells were compacted to pellets. Hot SDS-PAGE sample buffer, that is, 62.5 mM Tris, pH 6.8, 6% w/v SDS, 2% v/v glycerol, 5% v/v 2-mercaptoethanol, 0.0125% w/v bromophenol blue, and protease and phosphatase inhibitor cocktails (Sigma), was added to the pellets, and following sonication for 15 s, the samples were clarified by centrifugation and stored at −80°C until use. The lysates were separated on SDS-PAGE (gradient 5%–12.5%) and the proteins were transferred to Polyscreen PVDF membranes (Perkin Elmer, Thessaloniki, Greece). The membranes were blocked with 5% (w/v) nonfat dried milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20 (TTBS) buffer and incubated with the appropriate primary antibodies. After washing with TTBS, the membranes were incubated with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated goat secondary antibody (Sigma) and washed again with TTBS and the immunoreactive bands were visualized by chemiluminescence (LumiSensor HRP Substrate Kit, GenScript, Piscataway, NJ, USA) according to the manufacturer's instructions on a Fujifilm LAS-4000 luminescent image analyzer (Fujifilm Manufacturing, Greenwood, SC, USA).

2.7. Statistical Analysis. Results were expressed as mean values ± SEM. Differences in the presented values were evaluated by Student's t-test.

3. Results

3.1. DNA Synthesis Stimulation by Growth Factors. Primary bovine coccygeal AF cells cultured in 3D collagen type-I gels in the presence of a minute FBS concentration (0.1%) were arrested in the G_0/G_1 phase of the cell cycle as determined by flow cytometry after propidium iodide staining (not shown here). Under these conditions the cells exhibited intense DNA synthesis responses to PDGF-BB, bFGF, and IGF-I (Figure 1(a)). The highest response was elicited by PDGF (746%), followed by IGF-I (620%), and bFGF (301%). The responses to PDGF and IGF-I, however, did not differ statistically significantly, and only the response to bFGF was significantly lower compared to the other two growth factors. Similarly, bovine NP cells cultured in 3D collagen gels supplemented with CSA were also stimulated by the three growth factors with the same order of intensity (Figure 1(b)). In general, AF cell responses to each growth factor compared to the control were higher than the ones of NP cells; these differences however were not statistically significant.

The presence of CSA in the 3D culture system was observed to inhibit dose-dependently the basal (control) tritiated thymidine incorporation ($p < 0.01$ for both concentrations); this, however, positively affected NP cell proliferative response to PDGF as percentage of the control value (Figure 2(a)). More specifically, in the plain collagen gel (i.e., in the absence of CSA) PDGF-stimulated DNA synthesis was 308% ±20 compared to the control ($p < 0.01$), while in the presence of 250 and 500 μg/mL CSA PDGF stimulation was 458% ±41 and 412% ±24, respectively ($p < 0.01$ for both concentrations). There was no statistically significant difference between the two CSA concentrations used ($p = 0.09$); hence the concentration of 250 μg/mL was used in further experiments. Although the presence of CSA has physiological relevance only for NP, a similar experiment was performed with AF cells, and again CSA was found to suppress basal DNA synthesis without affecting PDGF stimulation (Figure 2(b)). Furthermore, a similar phenomenon was demonstrated in monolayer NP cell cultures using an alternative technique for assessing DNA synthesis, that is, BrdU incorporation (Figure 2(c) and Table 1).

3.2. Induction of Intracellular Signaling Pathways by Growth Factors. In conventional monolayer cultures, two pivotal signaling pathways have been found to mediate growth factor stimulation, that is, MEK/ERK and PI 3-K/Akt [15]. Here we report that, in 3D cultures of AF cells inside collagen gels, as well as in those of NP cells inside collagen gels supplemented with CSA, PDGF was found to phosphorylate both ERK and Akt (Figures 3(a) and 3(b)). Phosphorylation was induced rapidly and peaked at 1–3 hours, with the exception of pERK in AF cells, which peaked at 6 hours. bFGF induced immediately and intensely ERK phosphorylation, while its effect on Akt phosphorylation was less intense and it peaked at 3 hours (Figures 4(a) and 4(b)). On the other hand, IGF-I induced a less intense ERK phosphorylation peaking at 3–6 hours in the case of AF cells and at 1–3 hours in that of NP ones (Figure 5(a)). Finally, IGF-I-induced Akt phosphorylation was intense and sustained in both cell types (Figure 5(b)).

3.3. Response to Growth Factors in Relaxed Collagen Gels. The above data (Figures 1–5) were based in 3D collagen gels attached to the bottom and the wall of the culture plates, that is, stressed gels. Since there are reports from other cell types that relaxation of the collagen gel may affect the response

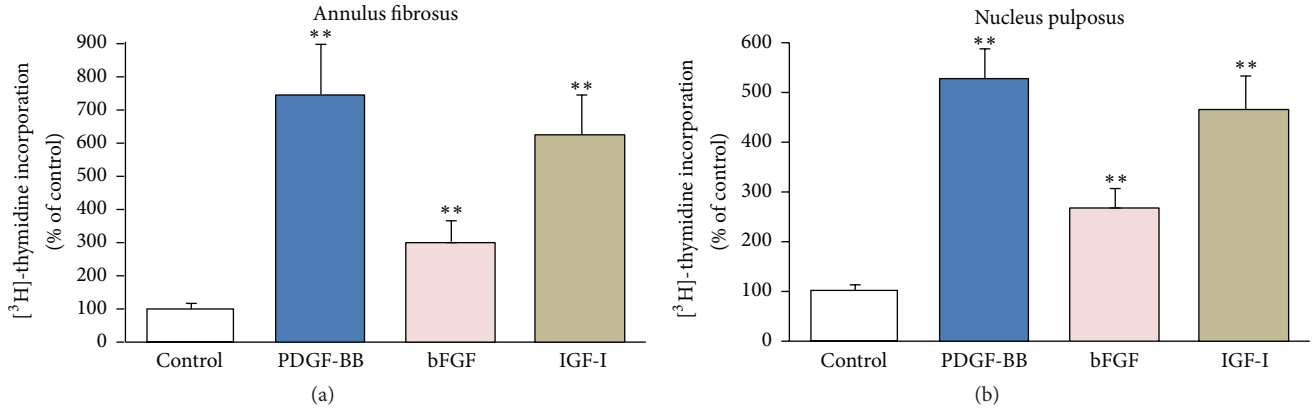

Figure 1: Effect of growth factors on DNA synthesis. Bovine AF (a) and NP (b) cells were cultured in collagen gels and collagen gels supplemented with CSA, respectively, and stimulated with PDGF (10 ng/mL), bFGF (5 ng/mL), and IGF-I (100 ng/mL) along with tritiated thymidine for 24 hours. Thymidine incorporation was assessed as described in Section 2. Values represent mean (±SEM) of three independent experiments. Asterisks (∗∗) indicate statistically significant differences compared to control ($p < 0.01$).

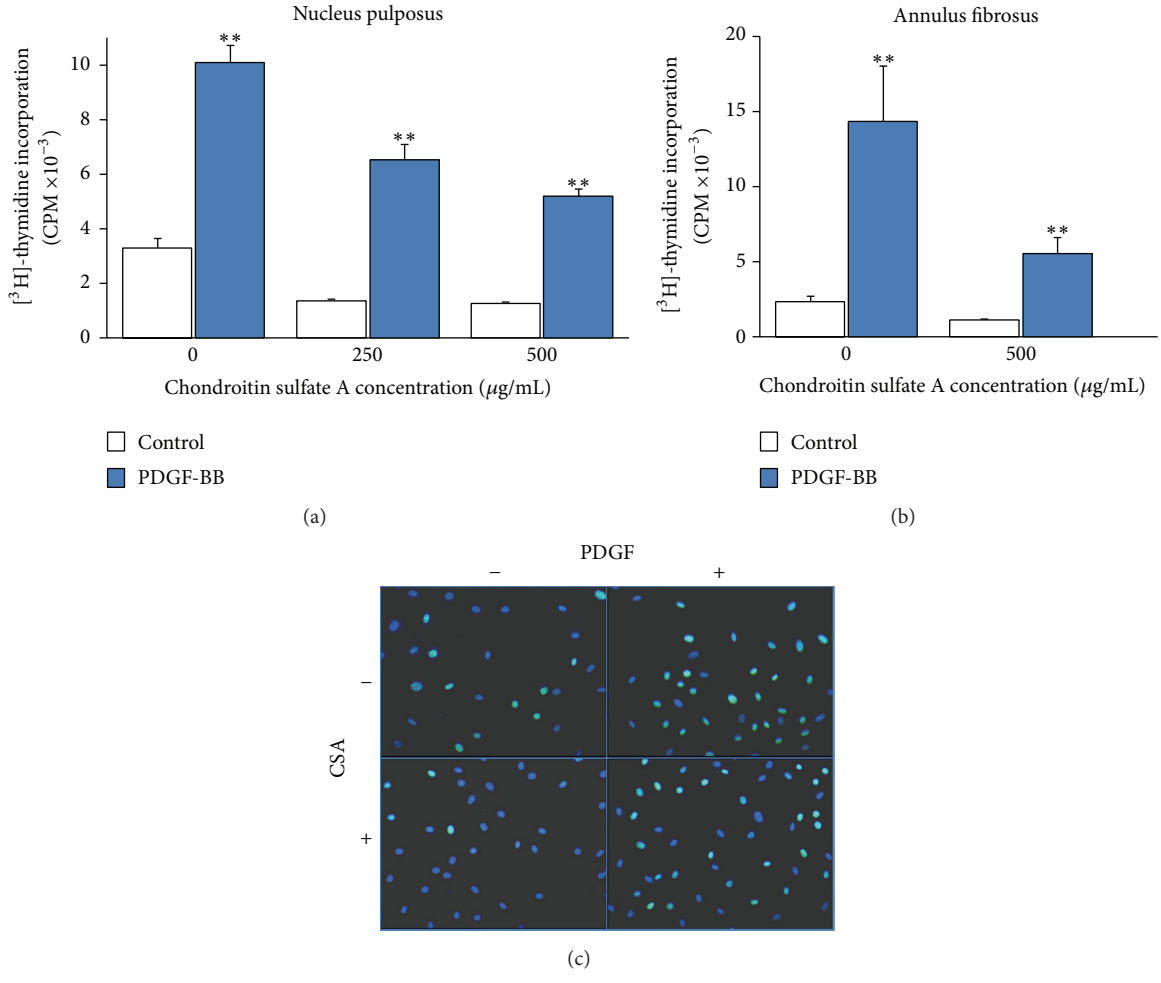

Figure 2: Regulation of PDGF-stimulated DNA synthesis by CSA. Bovine NP (a) and AF (b) cells were cultured in collagen gels in the presence of the indicated concentrations of CSA and stimulated with PDGF (10 ng/mL) along with tritiated thymidine for 24 hours. Thymidine incorporation was assessed as described in Section 2. Values represent mean ± SEM of three independent experiments. Asterisks (∗∗) indicate statistically significant differences compared to control ($p < 0.01$). In (c) monolayer cultures of bovine NP cells were stimulated with PDGF (10 ng/mL) in the presence or absence of 250 μg/mL CSA along with BrdU; incorporation of the latter was assessed as described in Section 2. Images were captured from representative fields with the FITC-filter and superimposed on images captured with the DAPI-filter.

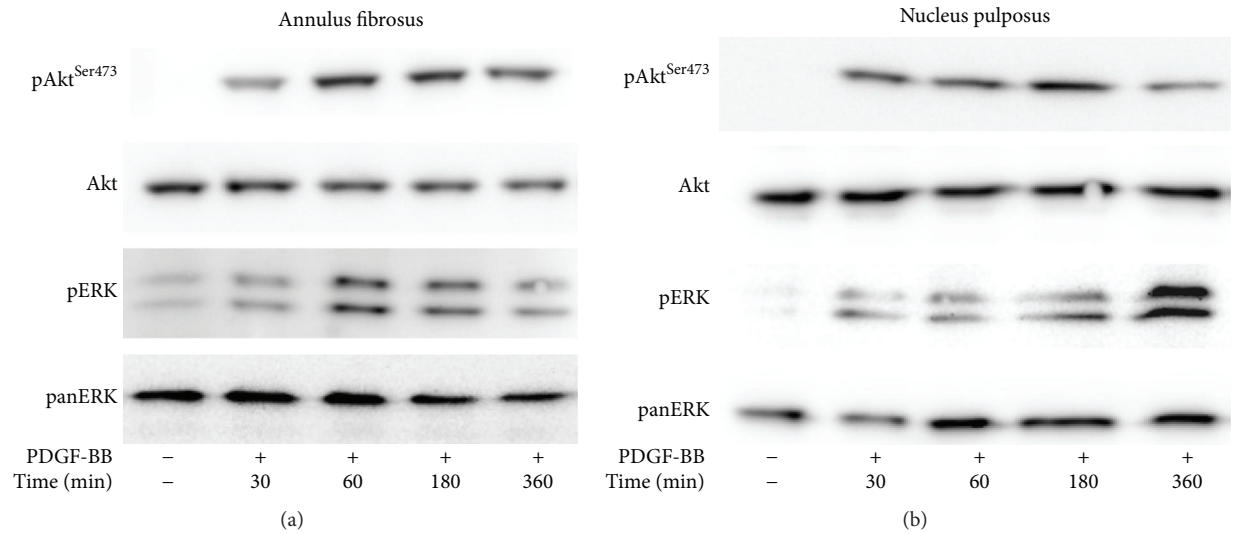

Figure 3: Activation of signaling pathways by PDGF. Bovine AF (a) and NP (b) cells were cultured in collagen gels and collagen gels supplemented with CSA, respectively, and stimulated with PDGF (10 ng/mL) for the indicated time intervals. Cell lysates were collected and subjected to SDS-PAGE and Western analysis as described in Section 2. One out of two independent experiments is depicted.

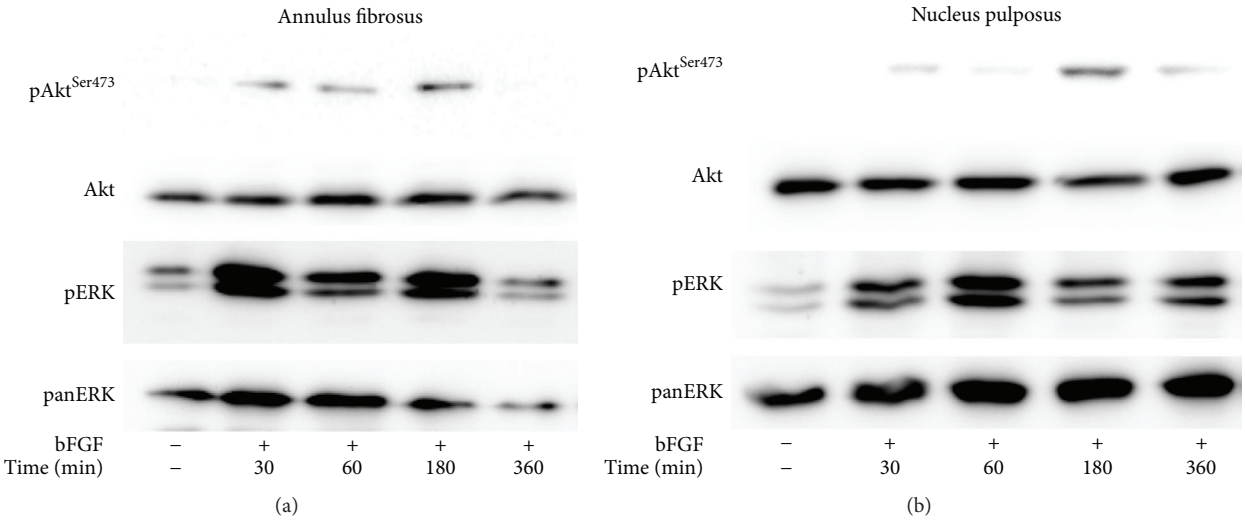

Figure 4: Activation of signaling pathways by bFGF. Bovine AF (a) and NP (b) cells were cultured in collagen gels and collagen gels supplemented with CSA, respectively, and stimulated with bFGF (5 ng/mL) for the indicated time intervals. Cell lysates were collected and subjected to SDS-PAGE and Western analysis as described in Section 2. One out of two independent experiments is depicted.

Table 1: Regulation of PDGF-stimulated DNA synthesis in monolayer NP cultures by CSA[a].

CSA concentration (μg/mL)	PDGF-BB concentration (ng/mL)	
	0	10
0	34.5% (±2.5)	58.1% (±2.1)
250	10.2% (±3.6)	46.2% (±10.2)

[a]Percentage of BrdU-positive nuclei (mean ± SEM from three independent cultures; three representative fields were counted in each culture).

to a growth factor [20], we have tested the response of AF cells cultured in floating collagen gels—detached from the bottom and the wall of the culture plate—to the three growth factors under study. As shown in Figure 6(a), AF cells in relaxed gels exhibit qualitatively similar proliferative responses to all three growth factors compared to cells in stressed gels (Figure 1(a)). Although stimulation in relaxed gels was higher than that in stressed gels for each growth factor, this difference was statistically significant only in the case of bFGF; more specifically the values were 1282% ±82 versus 746% ±150 ($p = 0.058$) for PDGF, 640% ±37 versus 301% ±66 ($p = 0.013$) for bFGF, and 942% ±37 versus 620% ±125 ($p = 0.144$) for IGF-I. As shown in Figure 6(b), in AF cells cultured inside relaxed collagen gels the ERK and Akt signaling pathways were activated by the three growth factors in an extent similar to that observed in the case of stressed gels (compare to Figures 3(a), 4(a), and 5(a)).

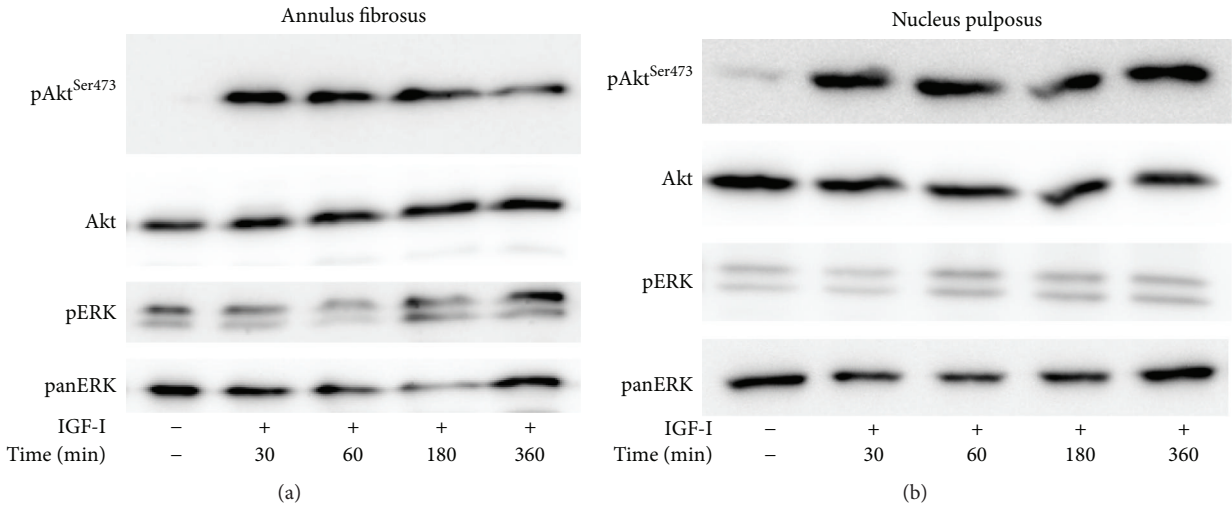

Figure 5: Activation of signaling pathways by IGF-I. Bovine AF (a) and NP (b) cells were cultured in collagen gels and collagen gels supplemented with CSA, respectively, and stimulated with IGF-I (100 ng/mL) for the indicated time intervals. Cell lysates were collected and subjected to SDS-PAGE and Western analysis as described in Section 2. One out of two independent experiments is depicted.

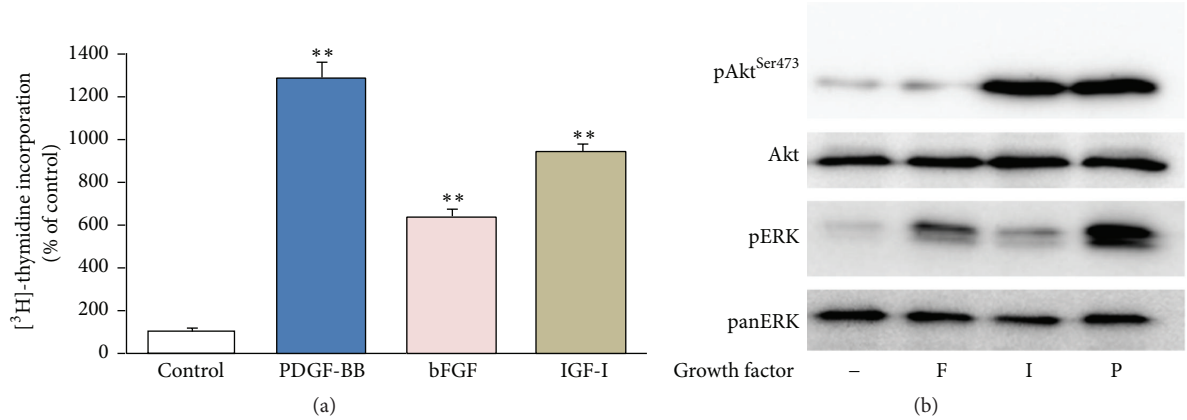

Figure 6: Effect of growth factors on AF cells cultured in relaxed collagen gels. Bovine AF cells were cultured in relaxed collagen gels and stimulated with 10 ng/mL PDGF-BB (P), 5 ng/mL bFGF (F), and 100 ng/mL IGF-I (I). In (a) tritiated thymidine incorporation was determined after 24 hours, while in (b) cell lysates were collected after 1 hour and subjected to SDS-PAGE and Western analysis. In (a) mean values (±SEM) of three independent experiments are shown ($^{**}P < 0.01$), while in (b) one out of two similar experiments is presented.

3.4. Contribution of Various Signaling Pathways to the Proliferative Response Elicited by Growth Factors. As shown above, the ERK and Akt pathways are activated by PDGF, bFGF, and IGF-I in AF and NP cells in 3D cultures. To determine whether these signaling pathways mediate the proliferative responses elicited by these three growth factors, DNA synthesis was monitored in the presence of the pharmacologic inhibitors PD98059 and wortmannin, blocking MEK/ERK and PI 3-K/Akt, respectively. The PI 3-K inhibitor LY294002 was also used for verification. Furthermore, the inhibitors calphostin-C and Y 27632, blocking Protein Kinase-C (PKC) and Rho-Associated Protein Kinase (ROCK), respectively, were also employed, since both signaling entities have been implicated in the maintenance of chondrocyte differentiation in 3D cultures [21, 22]. Since PDGF was found to be the most potent among the three growth factors (Figure 1), furthermore activating intensely both ERK and Akt (Figure 3), we studied the effect of the various signaling inhibitors on PDGF-induced DNA synthesis. In both AF and NP 3D cell cultures, PD98059 was the most potent inhibitor, followed by wortmannin and LY294002 (Figures 7(a) and 7(b)). Y 27632 was less potent, while calphostin-C had no effect at all. None of the inhibitors was blocking totally DNA synthesis induced by PDGF. Since PD98059 was the most potent among the inhibitors, we studied also its combinations with other inhibitors and observed that Y 27632 statistically significantly enhanced its inhibitory effect in AF cells (Figure 7(a)), and wortmannin did so in NP cells (Figure 7(b)). Collectively, these results indicate that, among the signaling pathways examined, the most important ones for mediating the growth stimulatory activity of PDGF are MEK/ERK and PI 3-K/Akt. These pathways were found to mediate also the proliferative effects of bFGF and IGF-I, both in AF cells (Figure 7(c)) and NP ones (not shown). The main difference with the case of

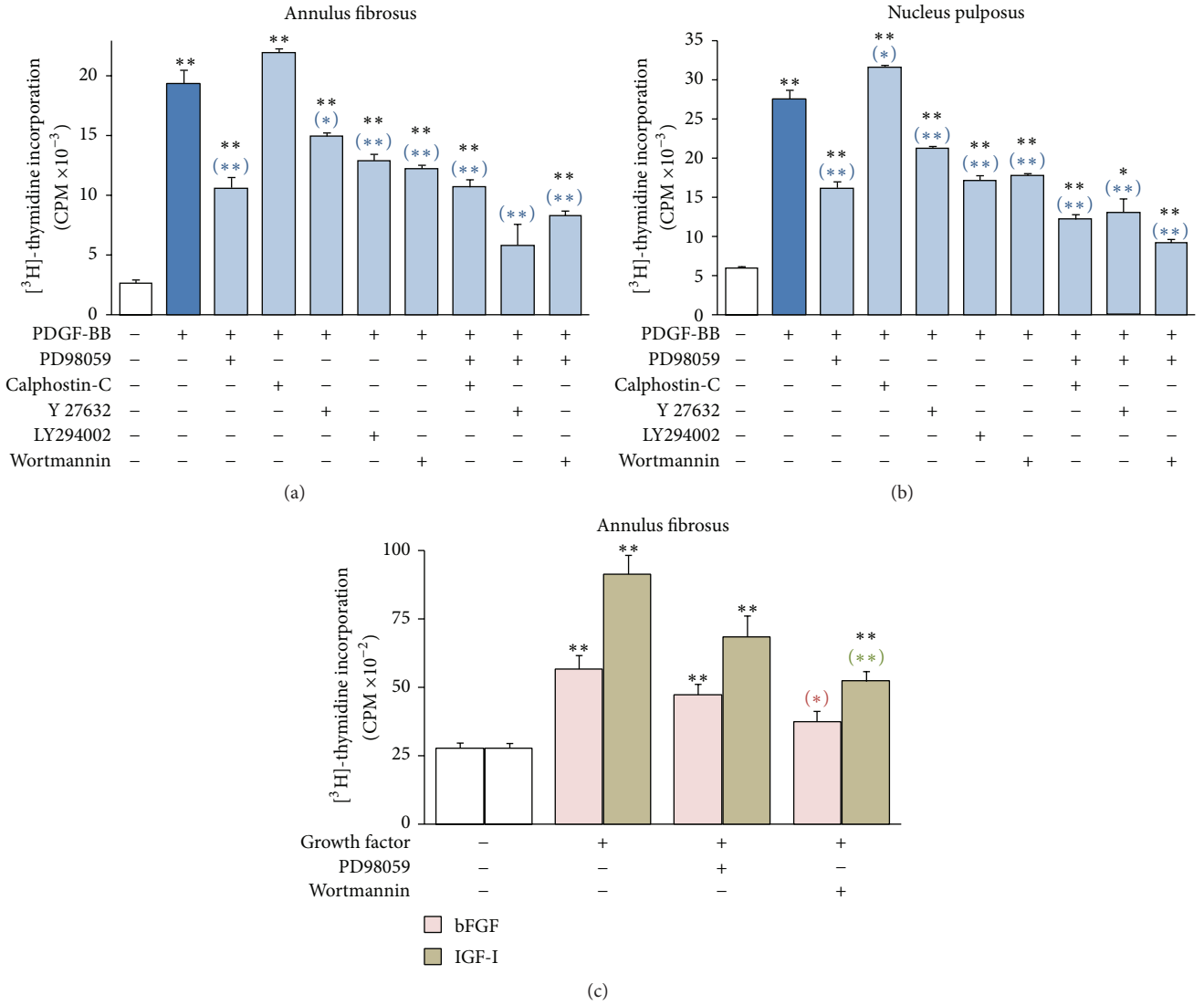

Figure 7: Inhibition of growth factor-induced DNA synthesis through blocking of signaling pathways. Bovine AF ((a) and (c)) and NP (b) cells cultured in collagen gels and collagen gels supplemented with CSA, respectively, were preincubated with PD98059 (25 μM), calphostin-C (100 nM), Y 27632 (10 μM), LY294002 (10 μM), wortmannin (100 nM), or the indicated combinations of them for 45 minutes. They were then stimulated with the indicated growth factors, and tritiated thymidine incorporation was determined after 24 hours, as described in Section 2. Mean values (±SEM) of three independent experiments are shown. Black asterisks indicate statistically significant differences compared to control, while blue, red, and green asterisks in parentheses indicate statistically significant differences compared to PDGF-BB, bFGF, and IGF-I, respectively ($^{**}p < 0.01$; $^{*}p < 0.05$).

PDGF was that wortmannin exerted a more potent inhibitory effect compared to PD98059 on the growth stimulation induced by bFGF and IGF-I.

4. Discussion

The aim of this study was to examine the growth response of IVD cells cultured in 3D culture systems—simulating the in vivo environment of the tissue—to PDGF, bFGF, and IGF-I. This effort was triggered by various observations in the literature indicating that IVD cells in monolayer culture have the tendency to dedifferentiate [23] similarly to chondrocytes [24]. Hence, many studies have been conducted in the very popular system of alginate beads, that is, a negatively charged gelatinous substance forming a 3D matrix [25] and resembling the negatively charged (due to the proteoglycans) milieu of the NP. On the other hand, this environment is far from the original extracellular matrix of the disc and most importantly is not very suitable for studying the proliferative response to growth factors, since IVD cells in alginate only marginally proliferate in response to serum [26]. Accordingly, collagen type-I gels offer a 3D environment containing one of the most abundant ECM constituents of the disc—at least regarding AF [4]—and they have been used extensively for the culture of chondrocytes, especially for tissue engineering purposes [27, 28]. Notably, 3D collagen gels have been shown

to permit the proliferation of chondrocytes, although not at the rate observed in monolayer cultures [29]. Furthermore, in order to better simulate the NP environment, we have added in the collagen solution the glycosaminoglycan chondroitin sulfate (CSA), an abundant constituent of NP proteoglycans [30].

The data presented here show that in the 3D environments we have employed both AF and NP cells exhibit an intense proliferative response to PDGF, bFGF, and IGF-I (Figure 1). Comparing these results with our previous study in monolayer cultures [15], one can see that the proliferative responses in the 3D environments are more intense than those in monolayers—as percentages of the respective control values—with the exception of PDGF acting on NP cells. This may be the result of the lower basal DNA synthesis levels observed in the control 3D cultures, at least regarding AF cells, in agreement with previous observations in other cell types concerning the growth restraining properties of the polymerized collagen [19, 31]. On the other hand, the more intense response to growth factors in the 3D environments may simply reflect an increased complexity of the network of activated signaling molecules compared to the monolayer cultures [32]. In general, the intensity of the proliferative responses to each growth factor in the 3D culture environments follows a similar pattern with that in monolayers, that is, PDGF > IGF-I > bFGF (Figure 1; [15, 16]).

The supplementation of collagen gels with CSA led to a further inhibition of basal DNA synthesis levels (Figures 2(a) and 2(b)), a fact observed also in monolayer cultures (Figure 2(c) and Table 1), in agreement with reports in the literature coming from different cell types [33, 34]. Furthermore, CSA has been shown to inhibit PDGF-induced proliferation of human lung fibroblasts [35] while stimulating that of human fibrosarcoma cells [36]. Nevertheless, in the present study and in a 3D culture environment, the proliferative responses of NP cells to PDGF (Figure 2) and to bFGF and IGF-I (not shown here) were maintained; actually they were higher in terms of percentages of the control values (see Section 3; Section 3.1). In the same direction, a growth promoting synergistic effect of chondroitin sulfate was observed for chondrocytes cultured in polyvinyl alcohol hydrogels and stimulated to proliferate with serum [37].

All three growth factors studied were found to induce two signaling entities of major importance regarding the regulation of cell proliferation, that is, ERK and Akt (Figures 3–5). Once again the patterns of ERK and Akt activation resembled those observed previously in monolayer cultures [15, 16], such as the intense activation of both pathways by PDGF, the less intense phosphorylation of ERK by IGF-I, and the much less intense activation of Akt by bFGF (Figures 3–5; see also Figure 6(a)). To our knowledge, this is the first study presenting data on the activation of signaling pathways in 3D cultures of IVD cells in response to these three growth factors. Phosphorylation of ERK and Akt in response to IGF-I has been shown previously in human articular chondrocytes both in monolayer cultures and in alginate beads [38].

Since mechanical stressing represents an integral part of IVD homeostasis [39], in an effort to identify any possible interference of the mechanical tensile forces with IVD cell responses to growth factors, we have tested cultures in relaxed collagen gels in addition to stressed ones. Interestingly, the responses of IVD cells to PDGF, bFGF, and IGF-I were found to be qualitatively similar both in stressed and in relaxed 3D collagen gels, in terms of DNA synthesis as well as intracellular signaling pathway activation (Figure 6). Our data may be at variance with reports from human skin fibroblast cultures, where the response to PDGF changes dramatically from stressed to relaxed collagen gels [20]; however in that case the two different culture environments correspond to two diverse phases of skin repair, that is, the granulation tissue versus the reconstituted dermis [40].

In our previous study conducted in monolayer cultures, we have shown that blocking the MEK/ERK or the PI 3-K/Akt signaling pathways results in a considerable inhibition of the growth stimulatory effects of PDGF, bFGF, and IGF-I on IVD cells, while the simultaneous inhibition of the two pathways completely blocks DNA synthesis induction by the three growth factors [15]. Our present observations from 3D cultures suggest that these two pathways are indeed very important for mediating the growth stimulatory effects of the three growth factors (Figure 7); however even the simultaneous blockade of MEK/ERK and PI 3-K/Akt did not completely inhibit growth factor-induced DNA synthesis, suggesting the involvement of more signaling molecules. The latter, however, does not belong to PKC, since its inhibition did not contribute more to the inhibitory effects of the MEK/ERK inhibitor PD98059 (Figure 7). ROCK, on the other hand, seems to be implicated only in the case of AF cells (Figure 7(a)), a fact possibly related to the intense tensile forces these cells are experiencing. Our future plans include the study of possible candidates for interfering with growth factor signaling in the IVD, such as several members of the integrin family, as well as focal adhesion kinase [41–44].

In conclusion, the present study indicates that IVD cells cultured in 3D organotypic gels respond to PDGF, bFGF, and IGF-I in terms of DNA synthesis stimulation, through the involvement of the pivotal MEK/ERK and PI 3-K/Akt signaling pathways. The proposed culture systems of collagen type-I gels for AF cells and CSA supplemented collagen gels for NP cells may prove useful for the in vitro proliferation and/or the delivery of cells aiming at the design of novel therapies for the regeneration of the degenerated IVD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Mutations in Biosynthetic Enzymes for the Protein Linker Region of Chondroitin/Dermatan/Heparan Sulfate Cause Skeletal and Skin Dysplasias

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Glycosaminoglycans, including chondroitin, dermatan, and heparan sulfate, have various roles in a wide range of biological events such as cell signaling, cell proliferation, tissue morphogenesis, and interactions with various growth factors. Their polysaccharides covalently attach to the serine residues on specific core proteins through the common linker region tetrasaccharide, -xylose-galactose-galactose-glucuronic acid, which is produced through the stepwise addition of respective monosaccharides by four distinct glycosyltransferases. Mutations in the human genes encoding the glycosyltransferases responsible for the biosynthesis of the linker region tetrasaccharide cause a number of genetic disorders, called glycosaminoglycan linkeropathies, including Desbuquois dysplasia type 2, spondyloepimetaphyseal dysplasia, Ehlers-Danlos syndrome, and Larsen syndrome. This review focused on recent studies on genetic diseases caused by defects in the biosynthesis of the common linker region tetrasaccharide.

1. Introduction

Chondroitin, dermatan, and heparan sulfate (CS, DS, and HS), classified as glycosaminoglycans (GAGs), are covalently attached to specific core proteins that form proteoglycans (PGs), which are ubiquitously distributed in extracellular matrices and on cell surfaces [1]. The classification of these polysaccharides has been based on their structural units: namely, the backbone of CS is composed of repeating disaccharide units of *N*-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcUA) (Figure 1), while DS consists of GalNAc and L-iduronic acid (IdoUA) instead of GlcUA (Figure 1). Both chains frequently exist as CS-DS hybrid chains in mammalian cells and tissues [2]. The backbone of HS consists of *N*-acetyl-D-glucosamine (GlcNAc) and GlcUA or IdoUA (Figure 1). These polysaccharides are modified by sulfation at various hydroxy groups and by the epimerization of GlcUA and IdoUA residues in the growing oligo- and/or polysaccharides [3–5]. These modifications provide structural diversity, thereby affecting a wide range of biological functions including cell proliferation, tissue morphogenesis, viral infections, tumor metastasis, and interactions with morphogens, cytokines, and growth factors [6–11].

The major component in cartilage is generally CS-PGs. Not only CS-PGs but also HS- and DS-PGs are expressed during bone development and regulate the maturation of chondrocyte [12]. CS side chains regulate bone morphogenetic protein and transforming growth factor-β signaling in the cartilage growth plate and chondrocyte columns [13]. HS or HS-PGs are known to be essential for Indian hedgehog signaling on cell surface by presenting to the receptor, Patched, in the growth plate [14]. DS-PGs, decorin and biglycan, play specific roles in all phases of bone formation including cell proliferation, matrix deposition, remodeling, and mineral deposition [15]. These observations suggest that defect in the biosynthesis of GAGs and core proteins of PGs might lead to disturbance of the skeletal development.

Genetic bone and skin disorders that are caused by mutations in the glycosyltransferases responsible for the

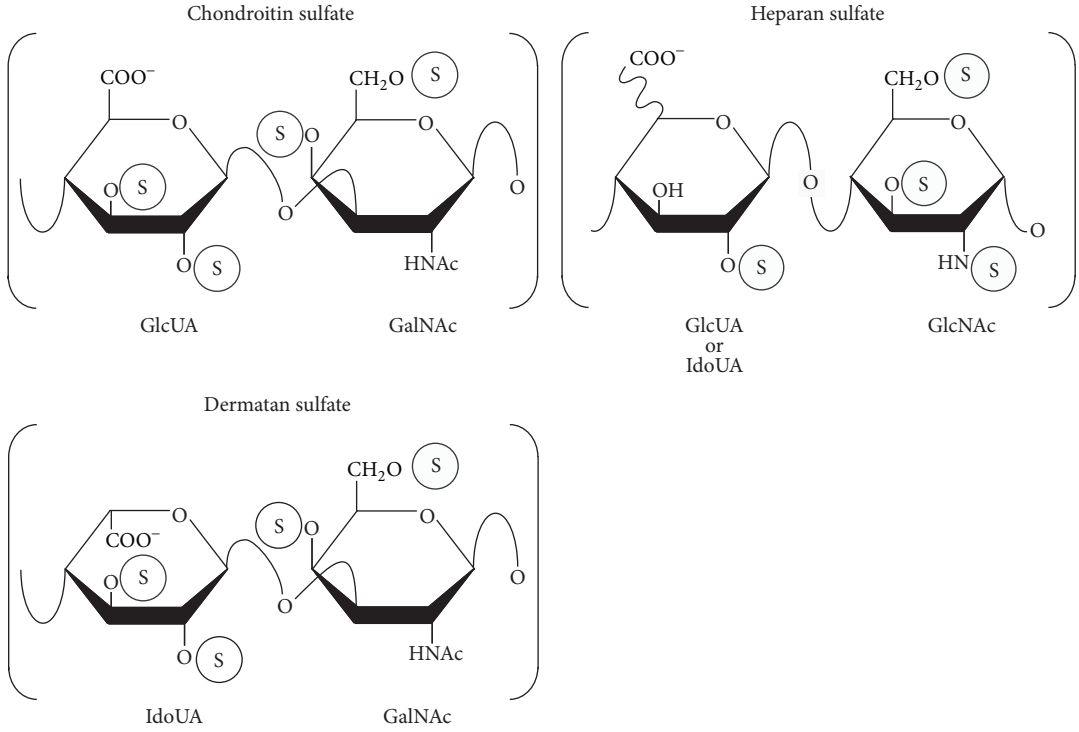

Figure 1: Typical repeating disaccharide units in CS, DS, and HS and their potential sulfation sites. The CS backbone consists of GlcUA and GalNAc, whereas DS is a stereoisomer of CS including IdoUA instead of GlcUA. The HS backbone consists of uronic acid and GlcNAc residues with varying proportions of IdoUA. These sugar moieties may be esterified by sulfate at various positions as indicated by the circled "S."

biosynthesis of CS, DS, and HS have recently been identified [6, 8]. This review focused on recent advances in the study of cartilage and connective tissue disorders caused by disturbances in the biosynthesis of the common linker region tetrasaccharide in CS, DS, and HS chains, which have been categorized as GAG linkeropathies.

2. Biosynthesis of CS, DS, and HS Chains

The repeating disaccharide regions of CS, DS, and HS chains are attached to serine residues in core proteins through the common GAG-protein linker region tetrasaccharide, -*O*-xylose-galactose-galactose-GlcUA- (-*O*-Xyl-Gal-Gal-GlcUA-) (Figure 2) [1]. β-Xylosyltransferase (XylT) catalyzes the transfer of a Xyl residue from uridine diphosphate-xylose (UDP-Xyl) to specific serine residues in the newly synthesized core proteins of PGs in the endoplasmic reticulum and *cis*-Golgi compartments, which initiates the biosynthesis of CS, DS, and HS chains (Figure 2 and Table 1) [16, 17]. Two Gal residues are added to serine-*O*-Xyl in the core proteins from UDP-Gal by β1,4-galactosyltransferase-I (GalT-I) and β1,3-galactosyltransferase-II (GalT-II), which are encoded by *B4GALT7* and *B3GALT6*, respectively [18–20]. β1,3-Glucuronosyltransferase-I (GlcAT-I) is encoded by *B3GAT3* and then transfers a GlcUA residue from UDP-GlcUA to serine-*O*-Xyl-Gal-Gal (Figure 2 and Table 1) [21].

The subsequent construction of the backbone of the repeating disaccharide region in CS chains [-4GlcUAβ1-3GalNAcβ1-]$_n$, is archived by six chondroitin synthase family members (Figure 2) [8, 9]. The formation of the backbone of the repeating disaccharide region of DS, -4IdoUAα1–3GalNAcβ1-, is achieved by DS epimerase, which converts GlcUA into IdoUA by epimerizing the C5 position of GlcUA residues during or after the construction of a chondroitin backbone [4]. On the other hand, exostosin (EXT) family members, including EXT1 and EXT2 as well as EXTL1, EXTL2, and EXTL3, have been shown to catalyze the formation of the HS-backbone, [-4GlcUAβ1-4GlcNAcα1-]$_n$ [8, 35] (Figure 2).

Thereafter, these polymer chains are maturated by sulfated modifications with various sulfotransferases, which transfer the sulfate group from a sulfate donor, 3′-phosphoadenosine 5′-phosphosulfate, to the corresponding hydroxyl groups at each sugar residue of the backbone, and by epimerization of the GlcUA residue with C5-epimerases [3–5, 8, 9, 35].

3. GAG Linkeropathy

3.1. XYLT1 and XYLT2. Mutations in *XYLT1* cause an autosomal recessive syndrome that is characterized by skeletal malformations such as a short stature and femoral neck, thickened ribs, plump long bones, characteristic facial features,

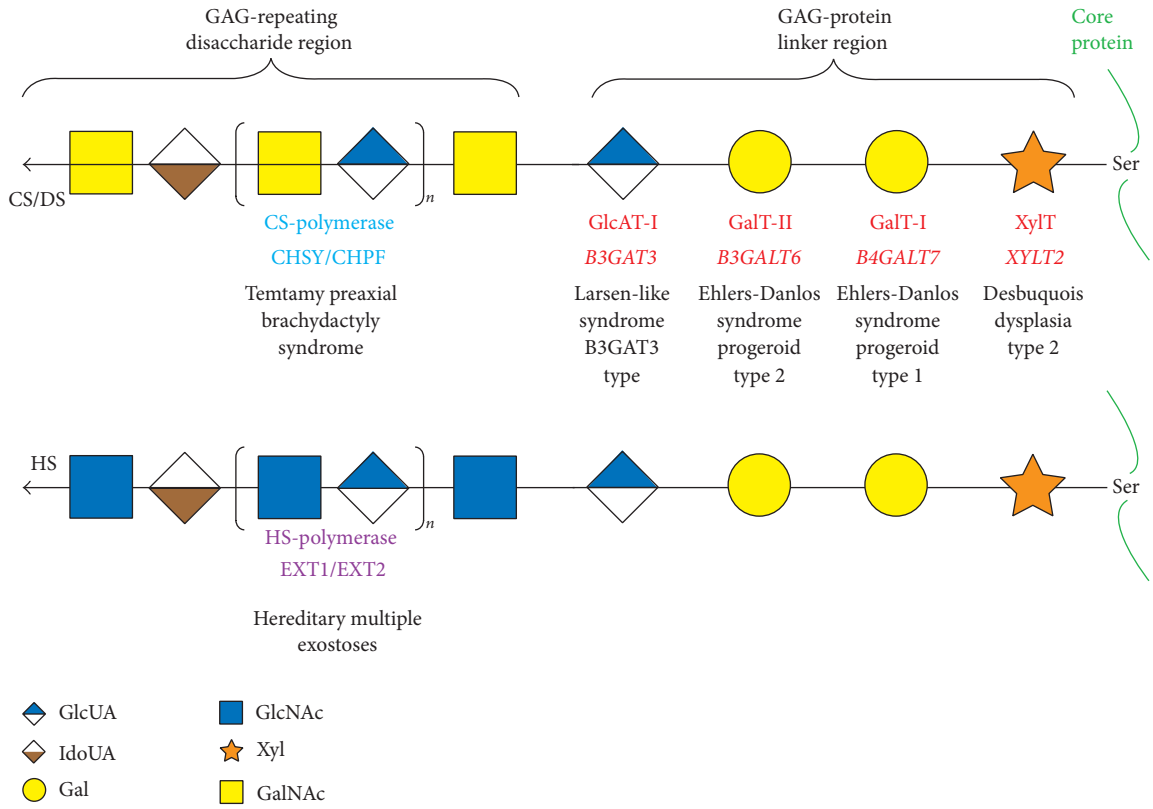

Figure 2: Biosynthetic assemblies of GAG-linker and GAG-disaccharide regions by various glycosyltransferases. All glycosyltransferases require a corresponding UDP-sugar, such as UDP-Xyl, UDP-Gal, UDP-GlcUA, UDP-GalNAc, and UDP-GlcNAc, as a donor substrate. After specific core proteins are synthesized, the synthesis of the common GAG-protein linker region, GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-, is initiated by XylT, which transfers a Xyl residue from UDP-Xyl to the specific serine (Ser) residue(s). The linker tetrasaccharide is subsequently produced by GalT-I, GalT-II, and GlcAT-I. These four enzymes are common to the biosynthesis of CS, DS, and HS. After the formation of the linker region, CS- and HS-polymerases assemble the chondroitin and heparan backbones, respectively. Each enzyme, its coding gene, and the corresponding inheritable disorder are aligned under the respective sugar symbols from the top of each line. CHSY, CHPF, and EXT represent chondroitin synthase, chondroitin polymerizing factor, and exostosin, respectively.

and intellectual disabilities [22]. A homozygous mutation in *XYLT1* was shown to result in the replacement of the amino acid, p.Arg481Trp, in the presumed catalytic domain, and the immature forms of decorin-PG without a DS side chain from the fibroblasts of the patient [22]. Furthermore, normal XYLT1 was found to be predominantly distributed in the Golgi apparatus of fibroblasts in a healthy control, whereas mutant XYLT1 was diffusely localized in the cytoplasm and partially in the Golgi in the fibroblasts of the patient [22].

Five distinct *XYLT1* mutations have been identified to date, including a missense substitution (p.Arg598Cys), nonsense mutation (p.Arg147X), truncated form mutation (p.Pro93AlafsX69), and two splice site mutations, resulting in Desbuquois dysplasia type 2, which presents severe clinical manifestations such as a short stature, joint laxity, and advanced carpal ossification [23]. In addition, the biosynthesis of high-molecular-weight CS-PGs, but not HS-PGs, was less in affected cells with these mutations in *XYLT1* than in healthy controls [23], suggesting that these manifestations may be caused by reductions in CS side chains, but not HS. These findings imply that XYLT1 mainly acts on serine residues in the core proteins of CS-PG, but not HS-PG, and that the functions of XYLT1 cannot be compensated by XYLT2.

Frameshift mutations in *XYLT2* cause an autosomal recessive syndrome that is characterized by osteoporosis, cataracts, sensorineural hearing loss, atrial septal defect, and learning difficulties similar to spondyloocular syndrome [24]. Two distinct mutations in *XYLT2* have been identified to date, including a homozygous frameshift duplication (p.Val232Glyfs*54) and deletion (p.Ala174Profs*35) [24]. XylT activity in serum was clearly less in affected individuals than in age-matched controls. Furthermore, fibroblasts from affected individuals were found to produce lower amount of CS and HS than those of controls [24]. These findings suggest that XYLT2 is also involved in PG assembly and is critical for normal development.

3.2. B4GALT7 (GalT-I). Mutations in *B4GALT7*, which encodes GalT-I, cause Ehlers-Danlos syndrome progeroid type 1, a disease that is characterized by an aged appearance, hypermobile joints, loose skin, craniofacial dysmorphism, a short stature, developmental delays, generalized osteopenia, and defective wound healing [25–27]. Ehlers-Danlos

Table 1: Biosynthetic enzymes of the GAG-linker region tetrasaccharide.

Enzymes (activity)	Coding genes	Chromosomal location	MIM number	Human genetic disorders (GAG linkeropathies)	Amino acid changes in disorders	References
Xylosyltransferase (XylT)	*XYLT1*	16p12.3	608124 615777	Desbuquois dysplasia type 2, short stature syndrome	Pro93Alafs*69; Arg147X; Arg481Trp; Arg598Cys; two mutations in splice site	[22, 23]
	XYLT2	17q21.33	608125	Spondyloocular syndrome with bone fragility, cataracts, and hearing defects	Ala174Profs*35; Val232Glyfs*54	[24]
β4-Galactosyltransferase-I (GalT-I)	*B4GALT7*	5q35.2-q35.3	130070 604327	Ehlers-Danlos syndrome progeroid type 1, Larsen of Reunion Island syndrome	Ala186Asp; Leu206Pro; Arg270Cys	[25–28]
β3-Galactosyltransferase-II (GalT-II)	*B3GALT6*	1p36.33	271640 615349 615291	Ehlers-Danlos syndrome progeroid type 2, spondyloepimetaphyseal dysplasia with joint laxity type 1	Met1?; Arg6Trp; Ser65Gly; Pro67Leu; Ala108Glyfs*163; Asp118Alafs*160; Met139Ala141del; Asp156Asn; Arg197Alafs*81; Asp207His; Gly217Ser; Arg232Cys; Cys300Ser; Ser309Thr	[29–31]
β3-Glucuronyltransferase-I (GlcAT-I)	*B3GAT3*	11q12.3	245600 606374	Larsen-like syndrome B3GAT3 type Multiple joint dislocations, a short stature, craniofacial dysmorphism, and congenital heart defects	Pro140Leu; Arg277Gln	[32–34]

syndrome is a heterogeneous group of heritable connective tissue disorders characterized by joint and skin laxity as well as tissue fragility [36]. Six major categories (classical, hypermobility, vascular, kyphoscoliosis, arthrochalasia, and dermatosparaxis types) and several minor categories (progeroid, musculocontractural, cardiac valvular, periventricular nodular heterotopia, and spondylocheirodysplastic types) have been identified to date [36]. The mutants GalT-I, p.Arg270Cys, p.Ala186Asp, p.Leu206Pro, and p.Arg270Cys, exhibited lower enzymatic activities than the wild-type [27, 37–40]. Furthermore, shorter CS and HS side chains on PGs as well as the partial lack of DS side chains on decorin and biglycan core proteins have been detected in cultured cells from these patients [27, 37–40].

A recent study reported that a homozygous mutation in *B4GALT7* (p.Arg270Cys) caused a variant of Larsen syndrome in Reunion Island in the southern Indian Ocean and is characterized by multiple dislocations, dwarfism, distinctive facial features, and hyperlaxity [28]. The symptoms of Larsen syndrome are congenital large-joint dislocations and characteristic craniofacial abnormalities including dislocations of the hip, elbow, and knee and foot deformities [41]. Therefore, Ehlers-Danlos syndrome (progeroid type 1) and Larsen syndrome (in Reunion Island) may share clinical spectra including joint dislocations.

3.3. B3GALT6 (GalT-II). Compound heterozygous mutations in *B3GALT6* encoding GalT-II cause Ehlers-Danlos syndrome progeroid type 2 [29, 30]. Three frameshift and two missense mutations have been identified in three patients [29]. Recombinant GalT-II mutant (p.Ser309Thr) exhibited significantly lower GalT-II activity than that of the wild-type enzyme [29]. Moreover, mutations in *B3GALT6* have also been shown to cause an autosomal-recessive disorder, spondyloepimetaphyseal dysplasia with joint laxity type 1, which is characterized by kyphoscoliosis, clubfeet, hip dislocation, elbow contracture, platyspondyly, and craniofacial dysmorphisms including a small mandible with a cleft palate, prominent eyes, and a long upper lip [29–31]. Skeletal and connective manifestations in Ehlers-Danlos syndrome progeroid type 2 and spondyloepimetaphyseal dysplasia with joint laxity type 1 largely overlap; however, these patients share no common mutations in *B3GALT6* [29]. The recombinant enzymes, p.Ser65Gly-, p.Pro67Leu-, p.Asp156Asn-, p.Arg232Cys-, and p.Cys300Ser-GalT-II, were found to exhibit significantly lower galactosyltransferase activities than that of wild-type GalT-II [29]. Although wild-type GalT-II is expressed in the Golgi, the mutant enzyme (p.Met1?), which affects the initiation codon, c.1A>G, is located in the cytoplasm and nucleus [29], suggesting that mislocalization of the mutant protein may cause GalT-II dysfunctions.

Furthermore, Malfait et al. identified three missense mutations and one frameshift mutation in *B3GALT6* in patients exhibiting various symptoms similar to those of Ehlers-Danlos syndrome and spondyloepimetaphyseal dysplasia with joint hyperlaxity [30]. Cultured fibroblasts from the affected individuals synthesized markedly less GAG and the DS side chain on decorin was absent. These findings indicated that not only the enzymatic activity, but also cellular localization of the mutant proteins affects the biosynthesis of CS, DS, and HS chains, leading to the abnormal development of skin, bone, and connective tissues.

3.4. B3GAT3 (GlcAT-I). A mutation (p.Arg277Gln) in the *B3GAT3* gene encoding GlcAT-I has been shown to cause Larsen-like syndrome [32, 33]. These patients predominantly have elbow dislocations with a bicuspid aortic valve in the heart as well as the characteristic manifestations of Larsen syndrome [32]. GlcA-transferase activity was markedly lower in the recombinant GlcAT-I mutant (p.Arg277Gln) and fibroblasts of these patients than in wild-type and healthy controls, respectively [32]. Moreover, fibroblasts from patients lacked the CS, DS, and HS side chains on PGs found in control cells.

A mutation in GlcAT-I (p.Pro140Leu) was recently identified from Nias island, Indonesia, in eight patients with a short stature as well as bone dysplasia including scoliosis, midface hypoplasia, dislocation of joints, broad ends of fingers and toes, and foot deformities [34]. The transferase activity of the recombinant GlcAT-I mutant (p.Pro140Leu) appeared to be lower than the wild-type [34]. Furthermore, the amounts of CS, DS, and HS from the lymphoblastoid cells of these patients were markedly lower than those of healthy controls [34]. These findings suggested that this mutation in *B3GAT3* (GlcAT-I) affected the biosynthesis of CS, DS, and HS side chains on PGs, leading to abnormal bone development.

4. Conclusions

Recent advances in genetic and glycobiological studies on connective tissue disorders have clarified the biological significance of GAG side chains and their linker region tetrasaccharides of PGs [6, 8]; however, the underlying pathogenic mechanisms remain unclear. Faulty biosynthesis of GAGs and PGs might affect assembly of matrix proteins and cell signaling during skeletal formation in GAG linkeropathies.

Various manifestations of GAG linkeropathies are clearly caused by mutations in the four glycosyltransferases responsible for the biosynthesis of the three kinds of polysaccharide chains, CS, DS, and HS, which are constructed on the core proteins of PGs. However, the clinical symptoms of GAG linkeropathies do not always agree among the different mutations of a glycosyltransferase nor the four distinct glycosyltransferases, XylT1, GalT-I, GalT-II, and GlcAT-I. These varieties of phenotypes may partially be due to distinct residual enzymatic activities, the cellular mislocalizations of mutant proteins, or partial compensation of the loss of function in each glycosyltransferase by other homologues, which may affect the quality of the GAGs yielded, that is, different lengths, numbers, and sulfation patterns of GAGs in the affected individuals.

Further comprehensive studies on the molecular pathogeneses of GAG linkeropathies are required for the development of design of new therapeutics for these diseases.

Abbreviations

B3GALT6: Beta-1,3-galactosyltransferase 6
B4GALT7: Beta-1,4-galactosyltransferase 7
B3GAT3: Beta-1,3-glucuronyltransferase 3
CS: Chondroitin sulfate
DS: Dermatan sulfate
GAG: Glycosaminoglycan
GalNAc: *N*-Acetyl-D-galactosamine
GlcUA: D-Glucuronic acid
HS: Heparan sulfate
IdoUA: L-Iduronic acid
PG: Proteoglycan
XYLT: Beta-xylosyltransferase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

A Comprehensive Review on Cyclodextrin-Based Carriers for Delivery of Chemotherapeutic Cytotoxic Anticancer Drugs

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Most of the cytotoxic chemotherapeutic agents have poor aqueous solubility. These molecules are associated with poor physicochemical and biopharmaceutical properties, which makes the formulation difficult. An important approach in this regard is the use of combination of cyclodextrin and nanotechnology in delivery system. This paper provides an overview of limitations associated with anticancer drugs, their complexation with cyclodextrins, loading/encapsulating the complexed drugs into carriers, and various approaches used for the delivery. The present review article aims to assess the utility of cyclodextrin-based carriers like liposomes, niosomes, nanoparticles, micelles, millirods, and siRNA for delivery of antineoplastic agents. These systems based on cyclodextrin complexation and nanotechnology will camouflage the undesirable properties of drug and lead to synergistic or additive effect. Cyclodextrin-based nanotechnology seems to provide better therapeutic effect and sustain long life of healthy and recovered cells. Still, considerable study on delivery system and administration routes of cyclodextrin-based carriers is necessary with respect to their pharmacokinetics and toxicology to substantiate their safety and efficiency. In future, it would be possible to resolve the conventional and current issues associated with the development and commercialization of antineoplastic agents.

1. Introduction

Poor aqueous solubility and rate of dissolution are the two critical factors that affect the formulation and development process of drugs and limit their therapeutic application [1]. The administration of drugs through different route especially of those, which are poorly soluble and belong to class II or IV of biopharmaceutical classification system, represents a major challenge [2]. Also, it is remarkable that most of the cytotoxic anticancer drugs belong to the BCS class IV which comprises substances with both low solubility in aqueous fluids and low apparent permeability [3]. Although several techniques like solubilization, [4, 5] cosolvency, [6] and solid dispersion [7–9] can enhance drug's solubility, bioavailability, and dissolution properties, these methods suffer from various disadvantages such as low drug loading and large dose. As an alternative, cyclodextrin (CD) complexation came into existence and presented a great interest [10, 11]. In 21st century, the concept of utilizing dual approach (cyclodextrins and nanotechnology) has emerged as a novel plan to tackle such formulation problems [12–14]. The purpose of this review is to discuss and summarize some of the potential findings and applications of cyclodextrin-based nanocarriers for effective delivery of anticancer drugs. This paper simultaneously explores the utility of cyclodextrin complexation and nanotechnology as unique approach for development of drug delivery system. Through this system, it would be possible to move the drugs of BCS classes II and IV into class I with certain limitations.

2. Cyclodextrins: Types and Complexation

Cyclodextrins are chemically and physically stable macromolecules produced by enzymatic degradation of starch. They are water-soluble, biocompatible in nature with hydrophilic outer surface and lipophilic cavity. They have the shape of truncated cone or torus rather than perfect cylinder because of the chair conformation of glucopyranose unit [15].

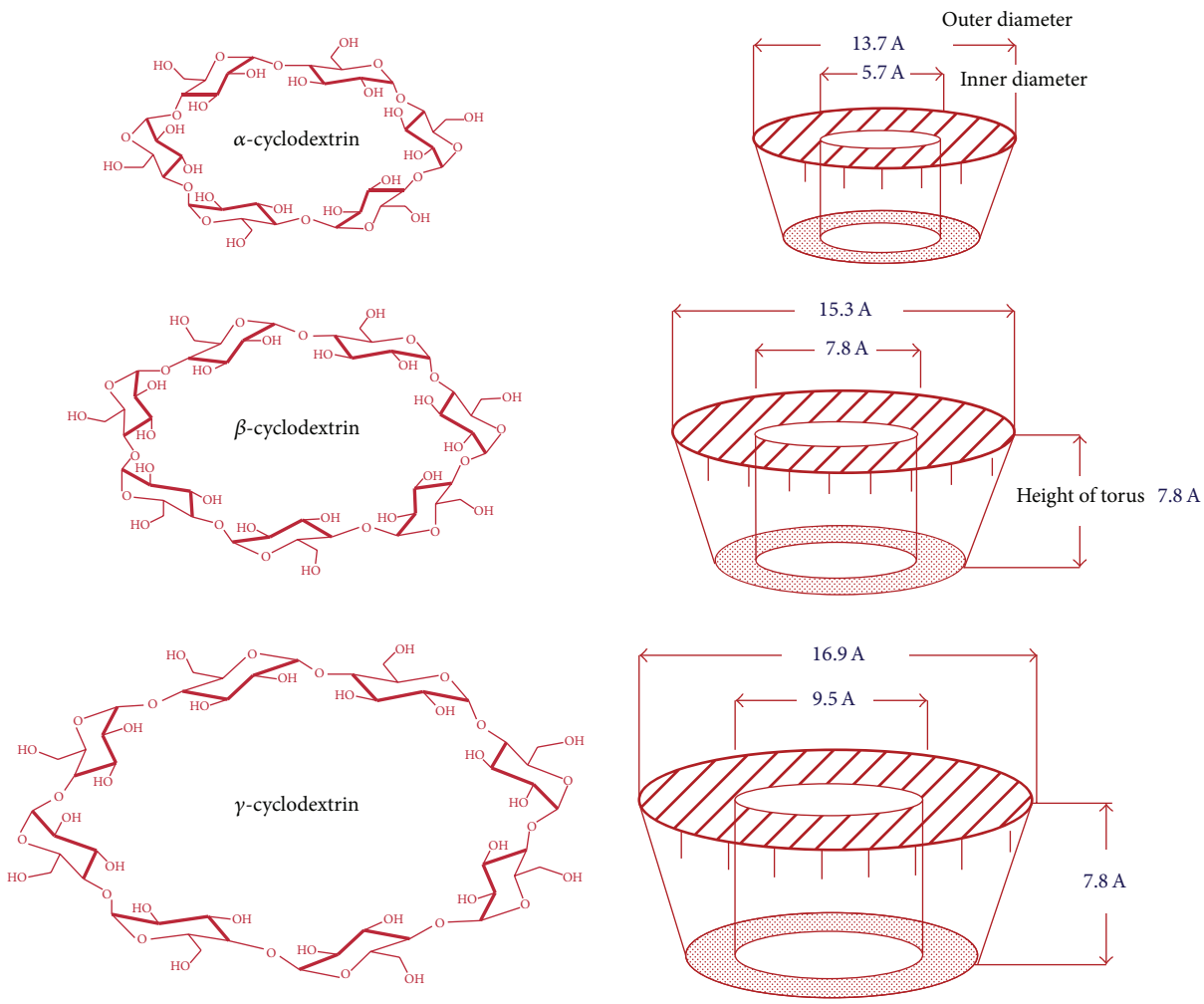

Figure 1: Structure and conformation of natural cyclodextrins.

Cyclodextrins are classified as natural and derived cyclodextrins. Natural cyclodextrinscomprise three well-known industrially produced (major and minor) cyclic oligosaccharides. The most common natural cyclodextrins are α, β, and γ consisting of 6, 7, and 8 glucopyranose units [16]. They are crystalline, homogeneous, and nonhygroscopic substances. Amongst these, β-cyclodextrin is ideal for complexation due to perfect cavity size, efficient drug complexation and loading, availability, and relatively low cost [17]. Figure 1 shows the structure and conformation of natural cyclodextrins. Various hydrophilic, hydrophobic, and ionic derivatives have been developed and utilized to improve the physicochemical and biopharmaceutical properties of drug and inclusion capacity of natural cyclodextrins [18–22]. Hydroxypropyl-β-cyclodextrin (HP-β-CD), randomly methylated-β-cyclodextrin (RM-β-CD), and sulfobutylether-β-cyclodextrin (SBE-β-CD) are mostly preferred for complexation [23, 24]. Polymerized cyclodextrins are high molecular weight compounds, either water-soluble or insoluble. They offer the advantage of amorphous state and complexation without toxic effects [25, 26]. The examples of polymerized cyclodextrins are soluble anionic β-cyclodextrin polymer, soluble γ-cyclodextrin polymer, and epichlorohydrin β-cyclodextrin polymer [27]. Due to superior solubilizing and complexing abilities exhibited these are nowadays most preferred for complexation [28, 29]. Table 1 enlists the characteristic features and properties of different types of cyclodextrins.

Inclusion complexes are formed when the "guest" molecule usually a drug is partially or fully included inside the "host's cavity" [30, 31]. Owing to the hydrophobic cavity, cyclodextrins as host offer the guest a suitable environment for interaction. The outer sphere of cyclodextrins is compatible with water, which allows hydrogen bonding cohesive interactions [32–34]. Due to this feature, CDs form inclusion complexes with a wide variety of hydrophobic compounds and change the physicochemical and biological properties of guest molecules [35–38]. These changes may enhance the therapeutic potential of drugs by diminishing their decomposition before they enter tissues and by altering how they enter tissue. The ability of a CD to form an inclusion complex is a function of steric as well as thermodynamic factors. The driving force for complexation involves the removal of water molecule from hydrophobic cavity and formation

Table 1: Characteristics features of different types of cyclodextrins.

Name of cyclodextrin	Solubility (mg/mL)	Mol. Wt. (Da)
Natural cyclodextrins		
Alpha cyclodextrin	145	972
Beta cyclodextrin	18.5	1135
Gamma cyclodextrin	232	1297
Chemically modified cyclodextrins		
Hydroxypropyl-β-cyclodextrin	≥600	1400
Sulfobutyl ether-β-cyclodextrin	≥500	2163
Randomly methylated-β-cyclodextrin	≥500	1312
Hydroxypropyl-γ-cyclodextrin	≥500	1576
Polymerized cyclodextrins		
Epichlorohydrin-β-cyclodextrin	>500	112000
Carboxy methyl epichlorohydrin beta cyclodextrin	>250	2000000–15000000

of Vander Waal forces, hydrophobic, and hydrogen bond interactions [39, 40]. The approach used for complexation is phase solubility study as described by Higuchi and Connors, which examines the effect of cyclodextrin (solubilizer/ligand) on the drug being solubilized (substrate).

3. Cyclodextrin Complexation with Anticancer Drugs

Chemotherapy for cancer, particularly for recurrent and metastasis disease, has limited therapeutic effect. Limited aqueous solubility (hydrophobicity), degradation in gastrointestinal fluids, insufficient *in vitro* stability (shelf life), low bioavailability, short *in vivo* stability (half-life), affinity for intestinal and liver cytochrome P450 (CYP3A4) and P-glycoprotein (P-gp) in the intestinal barrier, poor intestinal permeabilities, and strong dose dependent side effects of promising anticancer drug candidates have long been obstacles in treatment of cancer [41]. Lack of selectivity and short blood circulation time which cause various toxic side effects are also issues of major concern [42]. The narrow therapeutic index of some anticancer drugs and the fact that these cytotoxic drugs damage not only cancer cells but also normal and healthy tissue is a major challenge. Multidrug resistance, due to increased efflux pumps such as P-glycoprotein (Pgp) in the cell membrane, which transport most of anticancer drugs out of the cell, is also major problem [43, 44]. Thus, there is a need to develop such a delivery system, which combines safety, efficacy, and convenience. Cyclodextrins are competent enough to overcome certain forms of above associated drawbacks of anticancer drugs. The lack of efficient treatment has created the need to develop and implement novel technology based on combination strategy of cyclodextrin complexation and nanotechnology with a view to make the therapy more useful and acceptable. Figure 2 enlists the different approaches used for delivery of anticancer drugs.

The formation of inclusion complex with nontoxic agents leads to improvement in physicochemical properties of drug. Most of the anticancer drugs have been complexed with cyclodextrin and their derivatives to improve/enhance the solubility and stability, increase the bioavailability and dissolution, reduce the toxicity, and modify the physicochemical characteristics [45–57]. Complexation of doxorubicin with γ-CD and HP-γ-CD led to an increase in permeability across blood brain barrier, due to the disruption of the membrane [58]. Similarly, the β-CD-PEG folic acid conjugate increased the solubility of chlorambucil. Complexation of 9-nitrocamptothecin with HP-β-CD led to significant enhancement in antitumor activity with low toxicity [45]. Table 2 enlists the complexation of various anticancer drugs with cyclodextrins and their derivatives.

4. Cyclodextrin-Based Nanocarriers of Anticancer Drugs

The use of pharmaceutical carriers provides a loom, which is more time and cost-effective than new drug development [69]. Progress in nanotechnology and cellular/molecular biology has contributed to advancement in chemotherapy and gene therapy of cancer, optimistically avoiding the toxic doses of nonspecific agents. The development of new delivery system or new administration schedules offer less expensive, but more effective treatment with negligible/rare side effects [70]. Nanoparticles, with the size of about 100–10,000 times smaller than human cells, offer unique interaction with biomolecules, which may revolutionize cancer diagnosis and treatment. They have engorged surface area-volume ratio and can overcome both cellular and noncellular mechanisms of resistance, thereby increasing selectivity of drug towards cancer cell and reducing toxicity towards normal tissues [71]. Moorthi et al. described the use of nanotechnology to overcome the limitations associated with conventional cancer therapy. Some of the recent development includes the biodegradable methoxy poly (ethylene glycol)-poly (lactide) nanoparticles for controlled delivery of dacarbazine, cross-linked nanoparticles of cytarabine and microspheres of BCNU [72].

One of the major advantages that nanotechnology offers is targeted drug delivery to the site of disease. The aim of targeted therapy is to target the chemotherapeutics drugs to cancer cell, which ultimately reduce the side effects. Active

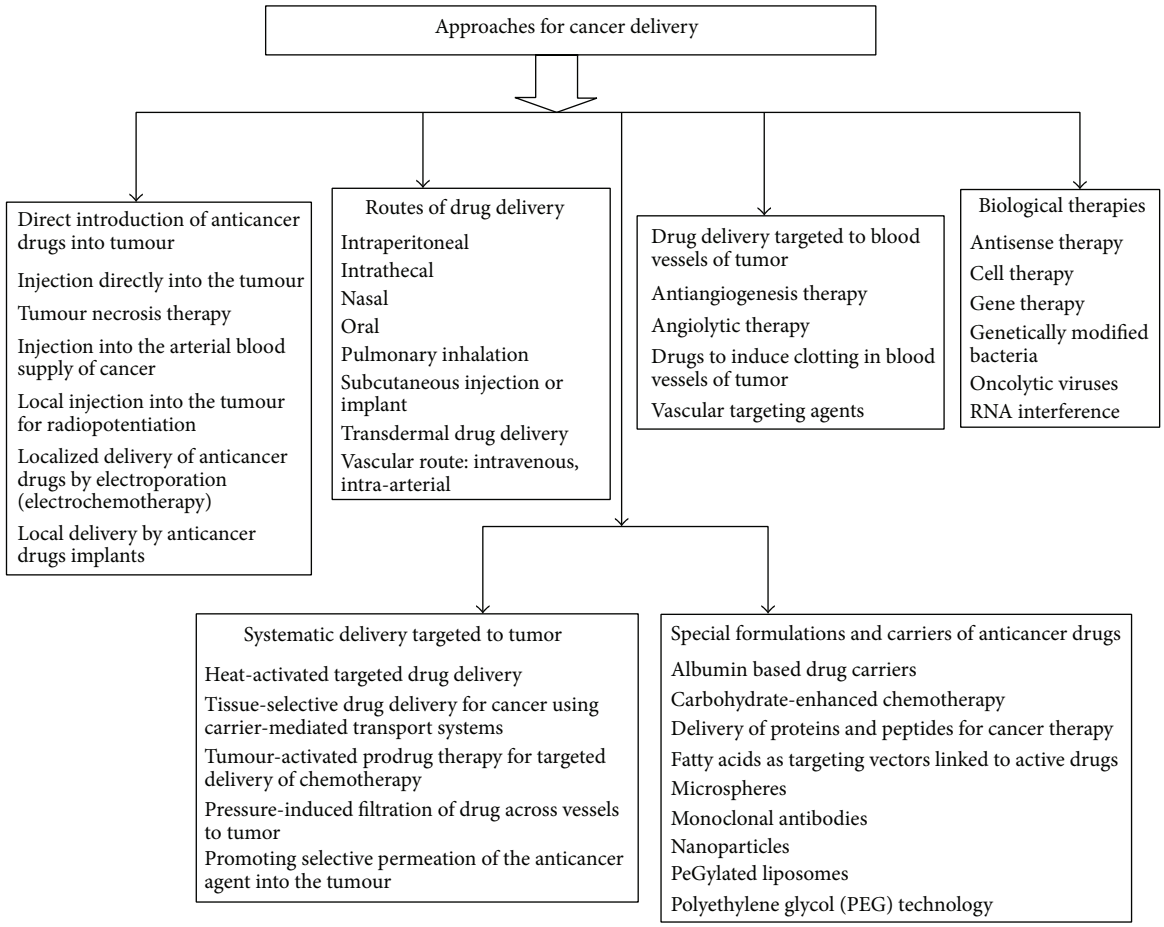

Figure 2: Approaches used for delivery of anticancer drugs.

targeting or affinity targeting involves conjugation of targeting molecules (like the antibodies, ligands, and nucleic acids) on the surface of nanoparticles with receptors overexpressed on a tumor cell surface [73]. In passive targeting, liposomes, macromolecular carriers, and nanoparticles exploit the EPR effect, which is a consequence of the increased vasculature permeability and decreased lymphatic function of tumors, to target the drug to the tumor [74]. Most of the cytotoxic chemotherapeutic agents are distributed nonspecifically throughout the body and affect both the normal and tumor cells [75]. The abnormal vascular structure plays a vital role for EPR effect in tumor for targeting of certain macromolecules at the tissues. The EPR effect is different from nontargeted, passive uptake of tiny molecules. Figure 3 shows the effect of EPR in tumor targeting. EPR is the mechanism by which a high-molecular-weight nontargeted drug/prodrug accumulates in tissues with increased vascular permeability such as in inflammatory sites and cancer. Nanotechnology based delivery system can reach tumor passively through leaky vasculature by EPR effect.

Cyclodextrin-based nanocarriers are prepared by utilizing the concept of dual approach, which involves combination of two different approaches in a single delivery system. This covers two aspects firstly; the anticancer drug is complexed with suitable cyclodextrin and secondly encapsulation of complexed drug into carrier. The encapsulation of chemotherapeutic anticancer drugs in specially designed, multifunctionalized cyclodextrin-based carriers would be a step toward their successful application in this challenging field. In drug delivery, entrapment of cyclodextrin complexed drug into nanocarriers increases the advantage of both of them. Encapsulation of cyclodextrin complexed drug into carriers will increase the drug loading capacity, entrapment efficiency, prolong the existence of the drug in systemic circulation, and reduces toxicity and provides controlled, sustained, or targeted release. Cyclodextrin adds value to the product. The unique feature of optimized drug-cyclodextrin complex includes lower aggregation; better ADME properties, rare toxicity, and are patient friendly [76].

Safety is important criteria for consideration before using cyclodextrins as pharmaceutical excipients. The safety and toxicity of cyclodextrins depend on the route of administration. When administered orally, cyclodextrins are not absorbed from gastrointestinal tract and thus are practically nontoxic. This is due to their bulky and hydrophilic nature. Any absorption, if it occurs, is by passive diffusion.

Table 2: Complexation of various anticancer drugs with cyclodextrin and their derivatives.

Serial number	Drug	Use	Cyclodextrin	Method	Outcome	Reference
1	9-Nitro camptothecin	Pancreatic cancer	HP-β-CD	Colyophilization	Significant improvement in antitumor activity and reduction in toxicity	[45]
2	Methotrexate	Melanoma	β-CD HP β-CD	Neutralization	Enhancement of aqueous solubility and bioavailability	[46]
3	Lonidamine	Prostate cancer	PM-β-CD	Physical mixture	Enhancement of solubility	[47]
4	Exemestane	Breast cancer	M β-CD	Kneading	Increase in solubility, improvement in bioavailability and dissolution	[48]
5	Vorinostat	Lymphoma	HP-β-CD, RM β-CD	Freeze-drying	Enhancement of bioavailability	[49]
6	Imatinib	Chronic leukemia	β-CD, RM β-CD	Freeze-drying	Enhancement of solubility	[50]
7	Doxorubicin	Lymphoma and leukemia	HP β-CD	Freeze-drying	Stability to acid hydrolysis and photodegradation	[51]
8	Cisplatin	testicular, ovarian, and cervical carcinoma	HP-β-CD	Freeze-drying and physical mixture	Increase in solubility, improvement in dissolution rate, and reduction of toxicity	[52]
9	Flutamide	Prostatic carcinoma	β-CD, HP-β-CD	Lyophilization	Enhancement of solubility and dissolution	[53]
10	Zerumbone	Colon and skin cancer	HP-β-CD	Freeze-drying	Improvement in solubility, stability, and bioavailability	[54]
11	Melphalan	Multiple myeloma and ovarian cancer	HP-β-CD	Freeze-drying	Stability against hydrolysis, solubility enhancement	[55]
12	Oridonin	Esophageal and cardiac cancer	β-CD	Freeze-drying	Enhancement of bioavailability	[56]
13	5-Fluorouracil	Cervical cancer	β-CD, HP β-CD	Colyophilization	Enhancement of solubility	[57]

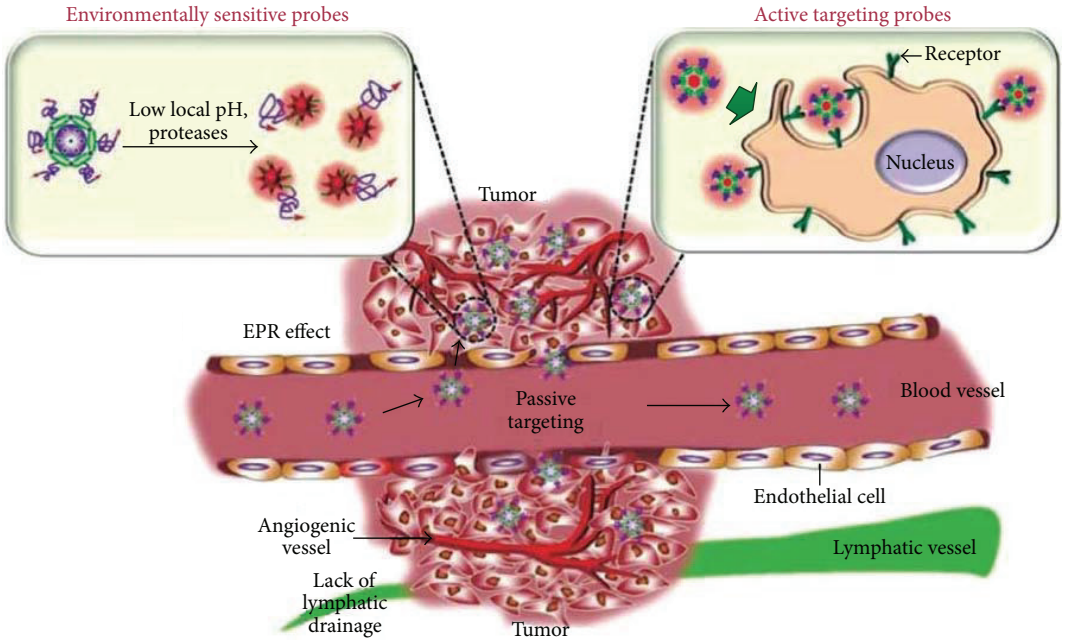

Figure 3: Role of EPR effect in tumor targeting.

Most of the hydrophilic derivatives of natural cyclodextrins like 2-hydroxypropyl-β-cyclodextrin and sulphobutylether-β-cyclodextrin are considered safe for parenteral administration. Higher dose of cyclodextrins may be harmful. Parenterally (intravenous) administered CDs disappear rapidly from systemic circulation and are renally excreted intact. When administered, CDs are distributed to the kidney, liver, urinary bladder, and various other tissues of the body. As per toxicity, profile of cyclodextrins is considered; several *in vitro* studies have reported the hemolytic effects of CDs although the toxicological implication of *in vivo* study is considered negligible. In 2008, Stella and He discussed the detailed safety and toxicity aspects of cyclodextrins with suitable examples [77].

Considering the regulatory aspects of various cyclodextrins, it is still evolving. Monograph of parent cyclodextrins (α and β) is available in various Pharmacopoeias like US Pharmacopeia, European Pharmacopeia, Japanese Pharmacopeia, and National Formulary. The monographs for various hydrophilic derivatives of cyclodextrins are also included in compendial sources like The Handbook of Pharmaceutical Excipients. Efforts are under process for inclusion of all other cyclodextrins in this list. Natural cyclodextrins are also included in US FDA list generally regarded as safe for their use as food additives [78]. The hunt for proficient and safe carrier to attain better drug availability at the targeted site has been an exigent area of research. Encapsulation of antineoplastic agents in carrier vesicles led to decrease in drug induced toxic side effects and increase in antitumor efficacy. Some of the cyclodextrin-based carriers of anticancer drugs are discussed with examples.

4.1. Cyclodextrin-Based Liposomes. Liposomes are concentric vesicles in which an aqueous volume is enclosed by membranous lipid bilayer. They entrap hydrophilic drug in the aqueous phase and hydrophobic drug in the lipid bilayer and retain drugs in route to their destination. In liposomes, cyclodextrin complexation competes with liposomal membrane binding, which tempers the potential benefit of complexation in prolonging hydrophobic drug retention [79]. The entrapment of water-soluble cyclodextrin-drug inclusion complexes in liposomes leads to accommodation of insoluble drugs in the aqueous phase of vesicles, increases the drug to lipid ratio, enlarges the range of encapsulation, allows targeting of complexes to specific sites, and reduces toxicity. Some of the examples are discussed as under Arima et al., in the year 2006, examined the antitumor effect of PEGylated liposomes of DOX complexed with γ-CD, administered through intravenous injection in BALB/c mice bearing colon-26 tumor cells. Results reflected retardation in tumor growth, increase in drug retention, and improvement in survival rate [59]. Dhule et al. 2012 evaluated the liposomal curcumin's potential against cancer models of mesenchymal (OS) and epithelial origin (breast cancer). The 2-HP-γ-CD/curcumin-liposome complex showed promising anticancer potential both *in vitro* and *in vivo*. In another study, the antiproliferative and cytotoxic activity of anticancer agent LPSF/AC04 in cyclodextrin complexed liposomes was enhanced [60]. Cui et al. 2011 developed stable PEGylated liposomal vincristine formulation with enhanced efficiency using sulfobutyl ether cyclodextrin as trapping agent. This formulation prolonged the circulation half-life from 43.6 to 70.0 hrs and reduced toxicity [80]. Thus, this strategy can incorporate several other cyclodextrin complexed anticancer drugs into liposomes and improve their retention inside vesicles.

4.2. Cyclodextrin-Based Niosomes. Niosomes are spherical lipid bilayers that can entrap water-soluble solutes within aqueous domains or alternatively lipid molecules within the lipid bilayers. They are prepared by hydrating the mixture of cholesterol and nonionic surfactants. These are vesicular systems similar to liposomes, biodegradable, biocompatible, and nonimmunogenic in nature and exhibit flexibility in their structural characterization. They are preferred over liposomes due to the higher chemical stability and economy. They alter the tissue distribution, cellular drug interaction and plasma clearance kinetics of the drug [81]. Oommen et al. 1999 entrapped methotrexate (MTX) complexed with β-cyclodextrin into niosomes. Complexation increased the entrapment efficiency and improved the anticancer activity. The entrapment efficiency was higher in the case of niosomes of MTX-β-CD complex (84%) compared to plain drug (67%). A relatively slow drug release of entrapped drug-complex from the vesicles compared to plain MTX encapsulated niosomes was observed [61]. This approach can manage the duration of action, in those cases where the dissociation constants of inclusion complexes can be modified.

4.3. Cyclodextrin-Based Nanosponges. Nanosponges are a class of microscopic particles with cavities of few nanometers wide, characterized by the capacity to encapsulate a large variety of substances that can be transported through aqueous media. Cyclodextrin-based nanosponges (NS) are prepared by cross-linking cyclodextrins (CD) with a carbonyl or a dicarboxylate compound as cross-linker. They are solid particles with spherical morphology with very high solubilizing effect and forms inclusion and noninclusion complexes with various drugs. The CD-cross-linker ratio can be varied to improve the drug loading and obtain a tailored release profile [82]. Swaminathan et al. 2010 prepared cyclodextrin-based nanosponges encapsulating camptothecin, for prolonging the shelf life and drug release. Camptothecin (CAM) has limited therapeutic utility because of its poor solubility, lactone ring instability, and serious side effects. The zeta potentials of cyclodextrin-based nanosponges of camptothecin were sufficiently high (−20 to −25 mV) leading to stable colloidal nanosuspension [62]. The *in vitro* studies indicated slow and prolonged drug release over 24 hrs and the cytotoxicity study showed that the formulations containing CAM were more cytotoxic than pure CAM. Mognetti et al. 2012 prepared paclitaxel-loaded β-cyclodextrin nanosponges, a water stable colloidal system avoiding the recrystallization of paclitaxel. The *in vitro* release studies showed that complete drug release was obtained within 2 hrs without an initial burst effect. The delivery of paclitaxel via nanosponges increased the amount of paclitaxel entering cancer cells and lowered the paclitaxel IC50, thereby enhancing its pharmacological effect [63].

Table 3: Cyclodextrin-based carriers of anticancer drugs.

Serial number	Drug	Cyclodextrin	Nanocarrier prepared	Outcome	Reference
1	Doxorubicin	γ-CD	Liposomes	Increased retention in tumor cells	[59]
2	Curcumin	HP-γ-CD	Liposomes	Improvement in therapeutic efficacy	[60]
3	Methotrexate	β-CD	Niosomes	Increased entrapment efficiency and solubility	[61]
4	Camptothecin	β-CD	Nanosponges	Improvement in therapeutic efficacy and reduction in toxic effects	[62]
5	Paclitaxel	β-CD	Nanosponges	Prolonged shelf life	[63]
6	Doxorubicin	β-cyclodextrin-based star copolymers	Micelles	Enhanced drug release	[64]
7	β-lapachone	α-CD	Polymeric millirods	Sustained drug release	[65]
8	Camptothecin	Amphiphilic β-CD	Nanoparticles	Prolonged drug release	[66]
9	Paclitaxel	Amphiphilic β-CD	Nanoparticles	Increased drug loading capacity	[67]
10	Docetaxel	Amphiphilic β-CD	Nanoparticles	Increased solubility and prolonged release	[68]

4.4. Cyclodextrin-Based Micelles. Micelles are self-assembled nanosized colloidal particles with lipophilic central part and hydrophilic covering. They have a single, central, and primarily hydrophobic zone or "core" surrounded by a hydrophilic layer or "shell." They have size in the range of about 5 to 2000 nm. They can entrap hydrophobic drugs at the core, which are transported at concentrations exceeding their intrinsic water solubility. The hydrophilic periphery of the micelle renders the polymer water-soluble and provides a tight shell around the drug-loaded core. This minimizes drug degradation and harmful side effects but increases bioavailability. Thus, these micelles can give a better therapeutic profile [83]. Drug is encapsulated into polymeric micelles either covalently or by physical encapsulation. Cyclodextrins as host are exploited for designing of self-assembled networks called polymeric micelles with guest molecules. A cyclodextrin micelle (CDM) contains derivatives of cyclodextrin including dimers, trimers, and polymers, incorporated as amphiphilic molecules and forms aggregate. Liu et al. 2012 formulated multifunctional pH-disintegrable micellar nanoparticles fabricated from asymmetrically functionalized β-cyclodextrin-based star copolymers covalently conjugated with doxorubicin (DOX), folic acid (FA), and DOTA-Gd moieties for targeted drug delivery. Results showed enhancement in drug release due to acid-labile feature of carbamate linkage [64].

4.5. Cyclodextrin-Based Polymeric Millirods. Site-specific, controlled release of cytotoxic agents from biodegradable polymer depots is a rising trend involved in cancer chemotherapy. Drug inclusion in a polymer depot also allows for potential tailoring of release kinetics, adding the benefit of being able to design the most efficacious delivery regimen. The incorporation of cyclodextrins into polymer millirods for complexing drugs significantly improves the drug release kinetics with various release patterns. The millirod consists of two functional compartments: an inner drug-loaded monolithic millirod as the drug depot and an outer NaCl-impregnated polymer membrane to control the release rate of the drug. The inner part of millirod permits the entrapment of drug particles in the matrix and provides sustained drug release [84]. Wang et al. 2006 prepared PLGA polymeric millirods for local delivery of β-lapachone. Complexation with HP-β-CD prevented the dissolution of drug and led to fast release (approximately 80%) after 2 days [65]. This data demonstrated the ability to tailor release kinetics via CD complexation and provided exciting opportunities for the use of millirods in intratumoral drug delivery.

4.6. Cyclodextrin-Based Nanoparticles. Nanoparticles are solid colloidal particles compos6ed of natural, synthetic, or semisynthetic polymers with size range from 1 nm to 1000 nm. Cyclodextrins increase the loading capacity of nanoparticles. Çirpanli et al. 2009 reported that the release of camptothecin was extended to 12 days with amphiphilic β-CD nanoparticles and 48 hrs with polymeric nanoparticles, showing the superiority of CD based nanoparticles over conventional polymeric nanoparticles [66]. The anticancer efficacy of amphiphilic CD nanoparticles was higher than that of PLGA/PCL nanoparticles loaded with CPT and its solution in DMSO. Agüeros et al. 2009 reported that the zeta potentials of the colyophilized nanoparticles of cyclodextrin complexed paclitaxel indicated stable colloidal dispersions within the range of −18 to −39 mV. Thus, paclitaxel encapsulation was higher with threefold increase in loading capacity [67]. Quaglia et al. 2009 prepared nanoparticles of the amphiphilic cyclodextrin heptakis (2-Ooligo (ethyleneoxide)-6-hexadecylthio-)-β-CD (SC16OH) entrapping docetaxel (Doc) to achieve prolonged drug release [68]. Table 3 covers the cyclodextrin-based nanocarriers for most of the anticancer drugs. Figure 4 shows the cyclodextrin-based carriers used in delivery of anticancer drugs.

4.7. Cyclodextrin Grafted Polymeric Nanocarriers. In this type of delivery system, natural cyclodextrins are grafted with polymers and then utilized for complexation with drugs. These complexes are then loaded into nanocarriers. Grafting is done by conjugating several units of cyclodextrins on polymer, to increase the binding ability of guest molecules

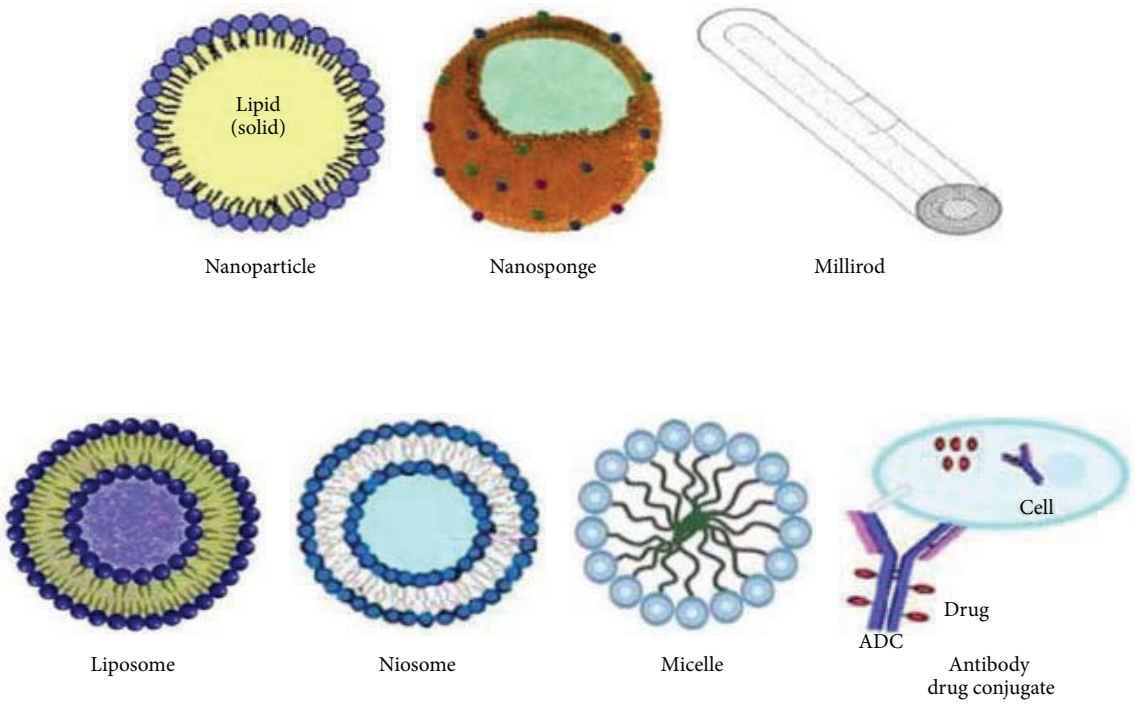

Figure 4: Cyclodextrin-based carriers for delivery of anticancer drugs.

and scale up the production. These polymers made of organized CDs provide the opportunity for drug molecule to associate at different levels within the nanostructure. For example, Zhang et al. 2011 designed a β-CD functionalized hyper branched polyglycerol (HPG-β-CD) of paclitaxel to achieve high drug loading capacity and aqueous solubility. The prepared nanoparticles had good biocompatibility and proved to be promising delivery system for hydrophobic drugs [85]. Recently, Zeng et al. 2013 prepared hollow nanospheres of camptothecin complexed with β-CD-graft-PAsp (β-cyclodextrin grafted with poly aspartic acid). Complexation led to improvement in aqueous solubility and stability of CPT. The nanoassemblies (nanospheres) of CPT-β-CD-graft-PAsp led to passive targeting of drug, sustained release, and decreased cytotoxicity [86]. These proved that cyclodextrin grafted polymeric nanocarriers can provide multifunctional properties for effective delivery of anticancer drugs.

4.8. Cyclodextrins Based Magnetic Nanoparticles. Cyclodextrins based magnetic colloidal nanoparticles are developed and fabricated to achieve targeted delivery of hydrophobic anticancer drugs. This is done by conjugation or grafting cyclodextrins with the drug through a linker moiety. For example, Badruddoza et al. in 2013 synthesized uniform nanocomposite for cell targeting of hydrophobic compounds. The magnetic particle Fe_3O_4 was encapsulated within the shell of silicon-dioxide using folic acid as the cell targeting ligand and β-cyclodextrin as the vehicle. These theranostic nanocomposite magnetic particles possessed multifunctional properties like cell targeting, fluorescence imaging, and delayed release [87]. Recently Sahu and Mohapatra in the year 2013 developed multifunctional magnetic fluorescent hybrid nanocarrier of 5-fluorouracil. Complexation led to increase in aqueous solubility and fluorescent carrier led to increase in stability and magnetic delivery of drug [88]. Several other hydrophobic anticancer drugs like curcumin [89], all-trans-retinoic acid, and methotrexate were also delivered using the same approach. In future, the concept of utilizing this approach of controlled release and drug targeting technology could provide more efficient and less harmful solution to conquer the limitations associated with conventional chemotherapy.

4.9. Cyclodextrin-Based siRNA (Short Interfering RNA) Delivery System. Small pieces of nucleic acid, known as siRNA, are one of the promising carriers for delivery of anticancer drugs. Free siRNA do not produce efficient and predictable therapeutic effect. They have biological half-life of less than an hour in human plasma. Thus, to increase the life span and improve the therapeutic efficacy, nonviral vectors are used. The therapy based on cyclodextrins and siRNA is currently under investigation for the treatment of cancer [90]. Synergistic therapeutic effect and regulation in tumor pathways are achieved by combining the drug with orthogonal therapeutic moieties like siRNA. Kim et al. 2011 synthesized cyclodextrin-modified dendritic polyamines for translocating siRNA and anticancer drugs suberoylanilide hydroxamic acid and erlotinib. The presence of β-cyclodextrins facilitated complexation and intracellular uptake of hydrophobic anticancer drugs, whereas the cationic polyamine backbone allowed electrostatic interaction with the negatively charged siRNA. Codelivery of siRNA-EGFRvIII and SAHA/erlotinib

in glioblastoma cells significantly inhibited cell proliferation and induced apoptosis compared to the individual treatments. Also, the DexAM complex possessed minimal cytotoxicity over a wide range of concentration and efficiently delivered siRNA, thereby silencing the expression of targeted genes. Thus, this study led to synergistic induction of apoptosis in brain cancer cells by targeted codelivery of siRNA and anticancer drugs [91]. Deng et al. 2011 synthesized star-shaped polymers for sustained delivery of methotrexate. This polymer exhibited higher transfection efficiency with low cytotoxicity in fibroblast cells. β-cyclodextrin was used simultaneously for the entrapment and sustained release of methotrexate [92]. Another important contribution in this field is the cyclodextrin-based nanoparticles designed to deliver siRNA agent to reduce the production of ribonucleotide reductase subunit M2 (RRM2). The study addresses the relevance of siRNA nanoparticles delivery for tumor-specific targeting. A/J mice bearing subcutaneous Neuro2A tumors were treated by intravenous injection of siRNA-containing nanoparticles formed with cyclodextrin-containing polycations (CDP). Results revealed that transferrin- (Tf-) targeted nanoparticles containing two different siRNA sequences slowed the tumor growth, whereas the nontargeted nanoparticles were considerably less efficient, when treated at the same dose. Another anticancer agent CALAA01, a targeted, self-assembling nanoparticles system based on CD complexed siRNA has been effective in phase I clinical trials for the treatment of solid tumors [93]. These examples highlight the recent advances in development of nanoparticles based on linear and cyclodextrin-based polymers for the treatment of cancer.

4.10. Cyclodextrin-Based Monoclonal Antibody Drug-Conjugate Approach. Many cytotoxic chemotherapeutic drugs have failed in clinical trials due to their extreme toxicity and lack of satisfactory therapeutic activity at the maximal tolerated dose (MTD). An important aspect of improving therapeutic activity of these anticancer drugs is to conjugate them with antibodies. Antibodies recognize the tumor-associated, cell surface antigens. Monoclonal antibodies (mAbs) represent a major class of agents currently used for cancer treatment. Therapeutic mAbs display better pharmacokinetic parameters, with moderate or no systemic toxicity [94]. The cytotoxic drugs covalently linked to a monoclonal antibody that recognizes a tumor-associated antigen is the antibody drug conjugate. This conjugate combines the selectivity, favorable pharmacokinetics, biodistribution, and functional activity of antibodies with the high cytotoxic potency of drug. A novel contribution in this regard is the antibody drug conjugate (ADC) of trastuzumab. Trastuzumab emtansine (T-DM1) is an ADC consisting of the anti-HER2 mAb trastuzumab (Herceptin) and the maytansinoid DM1 has been administered safely at therapeutically effective doses, despite HER2 being expressed on some normal tissues [95–98]. Genentech/Roche and their team are currently developing the ADC T-DM1 for patients with advanced HER2-positive breast cancer who have previously received multiple HER2-targeted medicines and chemotherapies [99]. Only few antibodies, tested against various tumor-associated antigens, such as rituximab, cetuximab, and panitumumab, are into the clinical practice [100]. Currently, researchers are interested in targeting of cancer stem cell specific antigens with antibody drug conjugate. The unique feature of monoclonal antibody drug conjugate is that it can overcome or minimize the multidrug resistance (MDR), which is a major obstacle in successful chemotherapy [101]. Thus, monoclonal antibody drug conjugate technology can deliver the powerful cytotoxic anticancer drugs with improved/enhanced pharmacokinetic properties. Earlier in 2003 Ikura et al. prepared A7 monoclonal antibody to β-CD conjugate using cell fusion technique, which served as useful tool for detection of β-cyclodextrin and its derivatives both quantitatively and qualitatively with its applications in various fields [102]. In 2003, Johns et al. investigated the antitumor efficiency of cytotoxic drugs in combination with (monoclonal antibody) mAb 806 by EGFR (epidermal growth factor receptor) inhibitor AG 1478. AG1478 is an inhibitor of EGFR tyrosine kinase, widely used in the laboratories [103]. However, it is insoluble in water and its various therapeutic potential is under process. Sulphobutyl ether β-cyclodextrin was used to solubilize AG1478. The investigation of John et al., 2003 proved that combination of cytotoxic drug with mAb can provide synergistic effect and enhanced antitumor efficiency. In future, this approach could be utilized for treatment of tumors.

4.11. Cyclodextrins Based Supramolecular Vesicles. Supramolecular complexes are formed by the combination of cyclic molecules with polymers (linear and/or branched). The use of supramolecular vesicles in drug delivery is expanding tremendously [104]. In case of cyclodextrin-based supramolecular complexes, the cyclic molecule is natural cyclodextrins and the polymers are PVA, PEG, PTA, PBA, PPG, and so forth; moreover, when the guest moiety (drug) is attached covalently to a host molecule (cyclodextrin) in a suitable way, an intra/intermolecular complex is obtained from supramolecular oligomer and polymer. Recently, the supramolecular self-assembly of cyclodextrins and polymers has led to the development of novel supramolecular hydrogels for drug delivery applications [105]. Nowadays supramolecular vesicles are developed for delivery of anticancer drugs. For example, a supramolecular linear-dendritic copolymeric micellar carrier of paclitaxel was developed using polystyrene and hyperbranched polyglycerols along with β-cyclodextrin [106]. This system provided higher loading capacity of paclitaxel. In another study, Paolino et al. 2012 investigated the potential of folate targeted supramolecular vesicular aggregate of GEM for treatment of solid tumors and breast cancer through *in vivo* models [107]. Thus, the supramolecular vesicles based delivery system could represent a novel approach by using self-assembling carriers and biocompatible polymers as potential for treatment of cancer.

5. Formulation Containing Cyclodextrin

An ideal drug delivery system should deliver the required amount of drug to the targeted site both efficiently and

precisely, for a desired time. For a drug molecule to be pharmacologically active, it must have some amount of aqueous solubility and lipophilicity in order to permeate the biological membranes through passive diffusion. The potency (effectiveness) and type of formulation determine the aqueous solubility of any drug [108]. A hydrophilic drug will not be able to partition from the aqueous exterior layer into lipophilic biomembrane. Improved therapeutic efficiency and targeted delivery of preexisting and new drugs can be achieved through carrier based novel drug delivery system, which is most suitable and approachable method for development of delivery system. Oral delivery of cytotoxic anticancer agents eliminates/minimizes the need for hospitalization, medical assistance, and infusion equipment. Cyclodextrins are used for encapsulation of lipophilic drugs and have demonstrated the ability to inhibit both PGP and cytochrome P450 localized on the surface of enterocytes [109]. To exploit the useful properties of both these above-mentioned features one of the anticancer drugs, PTX (paclitaxel) was loaded into poly (anhydride) nanoparticles after complexing with cyclodextrins. Interestingly, the relative oral bioavailability of PTX-cyclodextrin complex was 80%, which is remarkable for oral formulations. This example shows the promising use of cyclodextrins in oral formulations for delivery of anticancer drugs [110].

Cyclodextrins (CDs) form inclusion complexes with many drugs by trapping the molecule or part of it into the hydrophobic cavity. They are used as formulation additives and transdermal absorption promoters in topical delivery. Bilensoy et al. 2007 prepared vaginal gel formulation loaded with cyclodextrin complexed 5-fluorouracil, having thermosensitive and mucoadhesive properties. This ensures the longer residence of gel at the vagina, HPV-infection site, and the genital tract. Complexation provided favorable drug release with the reduction in side effects. This could be an efficient therapy for HPV-related diseases such as cervical cancer or genital warts with a lower dose.

Injectable formulations of water-insoluble drugs mainly consist of mixture of water, organic cosolvents, and surfactants. The use of organic solvents led to drug precipitation and cause pain, inflammation, and haemolysis [111]. Isotonic aqueous solution of cyclodextrins can replace the use of organic solvents and surfactants in injectable formulations. Among various cyclodextrins, HP-β-CD and SBE-β-CDs due to their high aqueous solubility and minimum toxicity are widely used in parenteral delivery. The advantage of using CDs in parenteral formulation includes solubilization of drug, reduction of drug irritation at the site of administration and stabilization of drugs unstable in aqueous environment, and so forth [112]. The use of cyclodextrins can reduce *in situ* irritation resulting from direct chemical irritancy of drugs, which cause phlebitis and pain at the site of injection. After intravenous injection, the drug is released rapidly and quantitatively from the complex upon dilution, followed by competitive replacement and binding to tissue and plasma proteins. Cyclodextrin exerts no effect on the pharmacokinetics of injected drugs. Ma et al. 1999 prepared injectable formulation of melphalan with SBE-β-CD and HP-β-CD. Through this formulation, the shelf life, solubility, and stability of the reconstituted melphalan were enhanced. Further, Oomen et al. 2010 prepared niosomal formulation for subcutaneous delivery of plumbagin. Complexation with β-cyclodextrin led to increase in aqueous solubility, stability, and efficacy and the niosome-entrapped drug-complex had improved anticancer activity as evidenced by the enhanced volume doubling time and growth delay [113]. Li et al. 2011 investigated the single and repeated-dose pharmacokinetics of injectable β-cyclodextrin-oridonin inclusion complex in rats. The results showed significant increase in the solubility and bioavailability of oridonin in rats. Finally, the hydrophilic cyclodextrin derivatives like hydroxypropyl-β-cyclodextrin and sulfobutylether β-cyclodextrin are relatively nontoxic and have minimal effect on the intrinsic pharmacokinetics of drugs. Thus, the formulations containing cyclodextrin are progressively being used during *in vitro* and *in vivo* screening of new anticancer compounds/drugs.

6. Current Status of Cyclodextrin

The impact of cyclodextrin-based nanocarriers and their therapeutics will likely accelerate in coming years. However, as these products move out of the laboratory and into the clinics, various federal agencies like FDA and US patents have to struggle in order to encourage the development of these products. This section highlights the contribution of those cyclodextrin-based therapeutic systems, which are under clinical trials or have been approved for human use [114]. Recently FDA approved the liposomal preparations of doxorubicin (Doxil), daunorubicin (DaunoXome), cytarabine (DepoCyt), and amphotericin B (Abelcet) which have proven to be attractive and less toxic alternatives to the conventional drug formulations and have opened new hopes for researchers. Until now, five (passively targeted) micelle products for anticancer therapy have been investigated in clinical trials, of which one has been granted FDA approval (Genexol-PM) for its use in patients with breast cancer [114]. Several liposomal formulations of conventional anticancer drugs are currently in phase I/II evaluation, including liposomal vincristine, platinum, mitoxantrone, all-trans-retinoic acid, and lurtotecan. There is a strong probability that these drug carriers will allow better administration of poorly soluble cancer drugs, enhance drug delivery and uptake in the tumor, and boost dose intensity, subsequently improving antitumor response. These recent reports favor the need of novel systems in this field.

In past 5 years, the significant contributions of cyclodextrins and their derivatives in drug delivery are discussed here, Dabur Pharma, one of India's leading manufacturers of anticancer drugs, launched Nanoxel, a novel drug delivery system for Paclitaxel, in 2007. This nanoscale drug delivery system is India's first indigenously developed nanotechnology-based chemotherapeutic agent throwing open a larger window for antitumor activity. In addition, the market for cyclodextrin-based drug delivery is remarkably increasing. Taj Pharmaceuticals (Mumbai based generic manufacturing Indian Pharmaceuticals Company) announced FDA approval for manufacturing of Piroxicam-beta-Cyclodextrin, Nimesulide-beta-cyclodextrin, Aceclofenac-beta-Cyclodextrin generic drugs

in regulatory market, and nonregulated market in the year 2009. The use of sulphobutylether-β-cyclodextrin is tremendously increasing in formulation and development. Recently FDA has approved five drug products containing captisol. They are Vfend I.V. Solution containing voriconazole, used in treatment of fungal infections, Nexterone containing amiodarone, used in ventricular arrhythmia, Geodon containing ziprasidone, used in schizophrenia, and so forth. This shows the wide use of cyclodextrins in pharmaceuticals. The number of the cyclodextrins-containing pharmaceutical products approved and marketed has been continuously increasing. Worldwide there are about 40 products or formulations containing various CDs, especially β-CD and its derivative. However, till date there is no single formulation containing anticancer drug with cyclodextrins in the market. This shows that there is a need to exploit the utility of cyclodextrins in the development of delivery system for anticancer drugs in the coming years.

7. Conclusion

Various nanotherapeutic approaches have been developed for delivery of anticancer drugs. But, still none of the available treatments for cancer is safe, effective, and able to treat the disease completely. Most of these are expensive, unacceptable, and inconvenient for long-term use or associated with significant toxicity. Thus, there is a need to move a step forward and utilize an advanced technology based on combination strategy, which exploits the advantages of both the systems, namely, cyclodextrin complexation and nanotechnology in single delivery system. The use of drug delivery systems, based on colloidal vesicles and macromolecular carriers (cyclodextrins), represents a promising and innovative strategy that enables effective therapy with minimum side effects. However, the major challenge is about the toxicity and pharmacokinetic study of these cyclodextrin-based carriers. Most of the information about toxicity is based on *in vitro* cell models. There are several studies concerning the interaction of nanoparticles within the body. But, there is no significant result for interaction of cyclodextrin complexed nanoparticles in the body. Also, there is a need to throw light onto the route of administration and the mechanism of elimination of these carriers. Another important parameter to be considered is the dose of anticancer drug and cyclodextrin used in the formulation. The extraordinary features of both these systems (cyclodextrin and nanotechnology) will simultaneously offer additional avenues to treat cancer successfully. Furthermore, there is a prerequisite to exploit the utility of these cyclodextrin-based nanocarriers using *in vivo* models for tumor targeting and toxicity studies of cancer like life-threatening disease. In future, cyclodextrins could be employed for modification of potent anticancer drugs to achieve effective treatment.

Conflict of Interests

Authors report no conflict of interests.

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Review Article

Heparan Sulfate Proteoglycans May Promote or Inhibit Cancer Progression by Interacting with Integrins and Affecting Cell Migration

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The metastatic disease is one of the main consequences of tumor progression, being responsible for most cancer-related deaths worldwide. This review intends to present and discuss data on the relationship between integrins and heparan sulfate proteoglycans in health and cancer progression. Integrins are a family of cell surface transmembrane receptors, responsible for cell-matrix and cell-cell adhesion. Integrins' main functions include cell adhesion, migration, and survival. Heparan sulfate proteoglycans (HSPGs) are cell surface molecules that play important roles as cell receptors, cofactors, and overall direct or indirect contributors to cell organization. Both molecules can act in conjunction to modulate cell behavior and affect malignancy. In this review, we will discuss the different contexts in which various integrins, such as α5, αV, β1, and β3, interact with HSPGs species, such as syndecans and perlecans, affecting tissue homeostasis.

1. Introduction

Metastasis is the ultimate result of cancer progression. There are several factors involved in the establishment of a metastatic site. These factors may be produced by cancer cells or by other cell types upon stimulation by a tumor. This review intends to present and discuss data on the relationship between integrins and heparan sulfate proteoglycans (HSPGs) in physiological conditions and during cancer progression. These two classes of molecules are deeply involved in cancer progression and can be found on the cell surface and the extracellular matrix (HSPGs only). We will focus on the mechanisms involving direct or indirect interaction between integrins and HSPGs, leading to altered cell behavior, such as cell adhesion, spreading, and cytoskeleton organization.

2. Integrins' Functions

Integrins are a family of cell surface transmembrane receptors, responsible for cell-ECM and cell-cell adhesion [1–3]. Due to their functions, integrins are considered fundamental for multicellular organism development. They have been expressed since early metazoans, although gene sequences may differ from group to group [4, 5]. Integrin functions by promoting cell adhesion, connecting the intra- with the extracellular space, leading to cytoskeleton arrangement, cell survival, differentiation, and growth [6–8]. These functions are relevant in embryo development and wound healing, as well as in various pathologies. Integrins are the main components of adhesion force generation, important for mesenchymal-like migration and collective migration, both relevant in cancer [9].

They are composed of two subunits, α and β. α subunit has eighteen isoforms, with molecular weights ranging from 120 to 180 kDa, while β subunit has eight isoforms ranging from 90 to 110 kDa [2]. Both subunits have only one transmembrane segment [2]. Different combinations of α and β subunits provide different affinities for ECM molecules. Each integrin dimer binds to different substrates; however, binding may be redundant among dimers. In the following lines, we will present the β subunits mentioned in this review.

β1 integrin pairs with 12 α subunits. They virtually occur in all vertebrate cells. β1 knockout mice are not viable because the embryo cannot perform implantation in the uterine wall. β2 integrin pairs with 4 α subunits. They only occur on white blood cells and are responsible for cell-cell interactions. β3 integrin is found on blood platelets and other cells. β4 integrins are major components of hemidesmosomes and their interaction with keratin filaments is relevant for cell-ECM adhesion [10, 11].

Integrin ligands are comprised of laminins, collagens, and the RGD motif, present in fibronectin and other proteins. Integrins interact with many other molecules on the cell surface, integrating intra- and extracellular compartments [12]. When binding to the extracellular matrix (ECM) for migration purposes, integrins cluster, forming a focal adhesion site, while when no clustering occurs, it is usually for activation of intracellular signaling. Finally, integrin trafficking is the main regulatory process of integrin availability on the cell surface [13].

Integrins present different activation states. Divalent cations affect integrins affinity and specificity; a balance between calcium, zinc, magnesium, and manganese may modulate integrin binding to its substrate [14–17]. Among the divalent cations, manganese has the most extreme modulating effect on integrin affinity for its substrates [14, 16]. Magnesium also activates integrins, while zinc will keep integrins in an inactive state.

Inside-out integrin activation is relevant for defense responses, especially when immune cells must bind to the endothelium or reach damaged areas during an infection or inflammation event [18–20]. Finally, integrins are also relevant in cell survival; lack of contact with the ECM leads to cell death [8]. Epithelial cells may have a different relationship than stromal cells as they differ in cell-cell and cell-ECM binding.

3. Heparan Sulfate Proteoglycans and Integrins

HSPGs play important roles during development; there are many examples of their ability to regulate cell growth, angiogenesis, tumor development, and other events. The average heparan sulfate (HS) chain is 50–200 repeating disaccharide units in length and is typically responsible for the majority of HSPGs functions, including protein-binding activity [21]. Different cell types express the same type of proteoglycan; however, these core proteins may present structurally different HS chains [22]; this suggests a finely regulated tissue-specific synthesis. Posttranscriptional and posttranslational modifications also influence proteoglycan variability [23]. Protein binding is usually mediated by HS chains, mostly by clustering basic amino acid residues with negatively charged regions of the glycan [24], but may also involve the proteoglycan core protein [25, 26]. Despite the fact that sulfate residues are largely responsible for the ionic interactions between HS and proteins (such as integrins and growth factors), hydrogen bonds and van der Waals interactions also play a significant role in HS-protein binding [27]. HS tridimensional conformation, defined by modifications during its biosynthesis, is a determinant feature of interactions between HSPGs and proteins [28]. ECM proteins, such as fibronectin, can synergistically bind integrins and syndecans and activate the cytoplasmic domain of this proteoglycan, controlling cell adhesion and motility by interacting with intracellular components [29, 30].

3.1. Syndecan-1. Syndecans are a family of transmembrane proteoglycans expressed throughout the organism [31]. All syndecans isoforms are regulated during development [32]. Many biological processes have been described as dependent on the interaction between syndecans and integrins. Their role in cell spreading, for example, is performed by exposing binding sites on fibronectin that can be recognized by integrins [33] or by modulation of integrin activation state [34].

Syndecan-1 is largely expressed in epithelia, contributing to the organization of adhesion molecules. This property has been shown in many works, whereas syndecan-1 influences integrin activation and cell arrangement.

Kato and colleagues have shown altered cell migration and reorganization after syndecan-1 loss; these changes could be associated with embryogenesis processes or even carcinoma development [35]. Studies on syndecan-1 role in cell adhesion by specific integrins show that this proteoglycan influences $\alpha 2\beta 1$, $\alpha V\beta 3$, and $\alpha V\beta 5$ integrins binding to collagen, vitronectin, and vitronectin/fibronectin, respectively [36–39].

Finally, integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ can also be activated by an inside-out system involving syndecan-1, via formation of a ternary complex: integrin-syndecan-insulin-like growth factor receptor, which leads to intracellular activation of the integrin by talin [40], promoting endothelial cell migration.

3.2. Syndecan-2. Syndecan-2 is not an integrin ligand, but it can interfere with its activation. In fibroblasts, syndecan-2 ectodomain binds to the protein tyrosine kinase phosphatase receptor, CD148, leading to an intracellular signal that induces β1 integrin-mediated cell adhesion [41]. Another study has shown that when syndecan-2 is shed from the endothelial cell membrane, it presents paracrine interactions with CD148, which leads to deactivation of β1 integrins, promoting an antiangiogenic effect [42]. Finally, it has been shown that syndecan-2 mediates adhesion to fibronectin in osteoblasts, its downregulation leads to reduced cell adhesion and spreading, and syndecan-2 downstream signaling molecule, ROCK, is also reduced in this context [43].

Overall, syndecan-2 indirect effect on integrin activation participates in the organization of different tissues; we will explore its role in tumor development in the following sections.

3.3. Syndecan-4. Syndecan-4 is an important component of focal adhesion and is involved in cytoskeletal reorganization. β1 integrin-mediated adhesion requires syndecan-4; nevertheless, there is no evidence of direct contact between these two molecules [44, 45]. This suggests a possible link with CD148, similar to syndecan-2 influence on cell adhesion [41]. The work by Chung and colleagues reinforces this link by revealing syndecan-4 interaction with CD148 as an important factor in the inhibition of T-cell activation [46].

Syndecan-4 has also a major role in regulating matrix structure and cell adhesion/migration during all stages of embryonic development and in most adult tissues. This phenomenon is strongly dependent on the interaction with β1 integrins, such as $\alpha5\beta1$, promoting focal adhesion assembly [47]. This assembly requires integrin turnover by endocytosis, which enables cell-ECM contact during migration [48, 49]. Another example of syndecan-4 influence on β1 integrins is the regulation of matrix structure described by Vuoriluoto and colleagues, whereas they show that syndecan-4 inhibits $\alpha2\beta1$ integrin-mediated collagen invasion [50]. Finally, Rønning and colleagues have shown that syndecan-4 cytoplasmic domain inhibits myogenesis, and its silencing during muscle differentiation leads to a higher expression of β1 integrin, possibly leading to the formation of focal adhesion [51]. Interestingly, when Carneiro and colleagues produced endothelial cell lines resistant to anoikis, these cells maintained β5 integrin levels but presented higher syndecan-4 expression [52].

All these lines of evidence indicate that syndecan-4 has important roles in cell migration, and, according to the developmental context, it may promote or inhibit cell adhesion/migration by mechanisms directly or indirectly associated with integrins.

3.4. Perlecan. Perlecan is ubiquitously expressed within the ECM and the basement membrane. This HSPG mediates cell signaling and controls cell differentiation, proliferation, and migration [21, 26]. Many developmental and homeostatic processes, like cartilage formation and wound healing, are dependent on its presence [53]. Perlecan knockout mice are not viable, resulting in early neonatal death due to abnormalities in ECM organization [54].

In brain infarcts, endorepellin, also known as perlecan domain V, has proangiogenic activity in brain microvasculature when in combination with $\alpha5\beta1$ integrins; this integrin dimer acts as a receptor for endorepellin and stimulates angiogenesis [55]. When endorepellin binds to $\alpha2\beta1$ integrins on endothelial cells, it blocks cell migration and angiogenesis by disassembling actin-stress fibers and focal adhesion [56]; this could be a control mechanism to avoid exaggerated angiogenesis. Endorepellin specific activity on endothelial cells was recently explained by the need of simultaneous expression of $\alpha2\beta1$ integrins and VEGFR2 found, so far, only in this cell type [57].

3.5. Agrin. Agrin is a multidomain ECM HSPG that was first discovered in neuromuscular junctions and other healthy tissues. It is widely expressed during development and plays a key role in the formation, maintenance, and regeneration of neuromuscular junctions [58]. It is known that αV and β1 integrins can act as receptors for agrin in muscle cells [59].

3.6. Collagen XVIII. Collagen XVIII is another HSPG with structural features of both collagens and proteoglycans [60]. Its C-terminal fraction, endostatin, interacts with $\alpha V\beta3$ and $\alpha5\beta1$ integrins, preventing endothelial cell migration and angiogenesis [61, 62].

These are examples of how HSPGs play key roles in integrin interaction with the ECM. Nowadays, many efforts are being made towards the elucidation of these interactions in order to develop better treatments to many diseases and malfunctions, especially cancer progression.

4. Heparan Sulfate Proteoglycans and Integrins in Cancer

HSPGs and integrins play important roles in cancer development. In this topic, we will describe the interactions between HSPGs and integrin and their effect on cancer progression.

4.1. Syndecan-1. Syndecans are one of the best portrayed HSPGs in studies on integrins and their engagement in cancer progression. Various interactions between these two classes of molecules modulate cell behavior in response to different signals [21, 63, 64]. Syndecan-1 association with integrins seems to generally induce tumor cell spreading and invasion, especially via interaction of its extracellular domain with $\alpha V\beta3$ and $\alpha V\beta5$ integrins [37, 38, 65]. Lines of evidence for these activities are described in the following lines.

MDA-MB-231 human breast carcinoma cells express syndecan-1 and syndecan-4. The signaling pathway associated with cell spreading in these cells seems to be dependent on $\alpha V\beta3$ integrin and syndecan-1, while syndecan-4 does not seem to be involved in this mechanism [37]. In addition, Beauvais and colleagues have shown that syndecan-1 ectodomain is specifically relevant for $\alpha V\beta3$ integrin binding to vitronectin in both MDA-MB-231 and MDA-MB-435 cell lines [38]. Syndecan-1 core protein and its complete form are not enough to establish adhesion sites on a collagen substrate by themselves; however, if this proteoglycan is presented in conjunction with $\alpha2\beta1$ integrins in MDA-MB-231 cells, adhesion is possible, and HS chains are mandatory for this interaction [66]. All these facts highlight the importance of syndecan-1 ectodomain in pathologic cell behavior.

Indirect interactions between syndecan-1 and integrins have also been described, such as the one between $\alpha6\beta4$ integrin and syndecan-1, an interaction mediated by human epidermal growth factor receptor 2 (HER2) that leads to tumor cell survival *in vitro* [67].

Syndecan-1 also affects other aspects of tumor progression, such as angiogenesis promotion during tumorigenesis. The work by Beauvais and colleagues shows that synstatin, a peptide derived from syndecan-1 active core protein, has antiangiogenic properties *in vivo* and *in vitro*, in addition to decreasing mammary carcinoma formation in nude mice. In this context, $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins are important to regulate angiogenesis [68], and, while syndecan-1 is necessary to regulate both integrins during angiogenesis and tumorigenesis, synstatin can cause the outbreak of this interaction [65].

Finally, syndecan-1 can also indirectly interfere with integrin by increasing integrin-ECM binding or by amplification of integrin signaling [37]. The work by Yang and colleagues shows that human myocardial fibroblasts secrete a fibronectin-rich ECM, which presents organized, parallel, fiber architecture. This fiber organization is dependent on syndecan-1 presence and is fundamental for the attachment and migration of breast carcinoma cells. This attachment probably occurs because this proteoglycan regulates the activity of several integrins, promoting fibronectin matrix assembly [69].

4.2. Syndecan-2. Many reports present syndecan-2 as an inhibitor of metastatic behavior. Munesue and colleagues have shown that low metastatic clones of Lewis lung carcinoma (LLC) cells present high syndecan-2 expression, while the highly metastatic clone does not. Induction of syndecan-2 expression in the highly metastatic clone mimics the low metastatic clone behavior, with the formation of actin-stress fibers mediated by $\alpha 5\beta 1$ integrin that, ultimately, will reflect on low invasive capacity [70, 71].

Syndecan-2 shedding has also an antiangiogenic effect in endothelium. CD148 interacts with shed syndecan-2 in endothelial cells, causing changes in $\beta 1$ integrin activation state, which leads to angiogenesis inhibition, affecting tumor growth [72]. This fact could be taken into account as an important way to develop novel therapies for diseases strongly dependent on angiogenesis for progression.

On the other hand, syndecan-2 may also promote invasiveness, as seen in MDA-MB-231 cells, whereas this proteoglycan has an important role in cell spreading and adhesion, leading to invasiveness and preserving a malignant phenotype, dependent on Rho GTPases, which regulates the actin cytoskeleton [73].

4.3. Syndecan-4. Syndecan-4 physiological role in focal adhesion formation can also be translated into tumor progression. Many reports have shown its importance for tumor cell survival, adhesion, and migration in the various conditions faced by a tumor cell during cancer progression, such as the ability to bind to the endothelium or thrive in hypoxic conditions.

Syndecan-4 phosphorylation was found to have an important role in the control of integrin recycling. This proteoglycan can control $\alpha V\beta 3$ integrin trafficking to the plasma membrane, promoting sustained focal adhesion in healthy mouse cells. The essential molecules in this process, as well as integrin recycling events and integrin expression changes, are found in processes like tumor invasion, demonstrating a route that can be further studied in cancer progression [74–77].

Syndecan-4 has also been associated with the metastatic phenotype; analyses of renal cell carcinoma samples and the highly metastatic tumor cell line KP1 have revealed an association between aggressive phenotype and high expression of tissue transglutaminase (TG2) and syndecan-4. This fact can be associated with syndecan-4 and $\alpha 5\beta 1$ integrin interactions [78, 79].

It was recently discovered that the endothelial surface molecule Thy-1 (CD90) is important for B16/F10 melanoma cells adhesion to endothelium via $\alpha V\beta 3$ integrin, favoring metastasis in an *in vivo* model [80]. Likewise, syndecan-4 promotes A375 melanoma cells binding to the endothelium by participating of a ternary complex with $\alpha 5\beta 1$ integrins and Thy-1. This complex promotes a strong interaction between the tumor cell and the endothelium, which is suitable for downstream mechanosignaling [81].

Cancer cells change their expression profile when challenged in hypoxic conditions. Koike and colleagues have shown that hypoxic human colon cancer cells remarkably overexpress syndecan-4 and $\alpha 5$ integrin, which are important cell-adhesion molecules involved in the enhanced adhesion of cancer cells to fibronectin [82].

Overall, syndecan-4 is a versatile molecule regarding tumor progression and more studies on its roles in cell physiology and the changes that accompany an invasive phenotype are needed for further advances in this field.

4.4. Perlecan. High expression of perlecan was found in some carcinomas, suggesting its involvement in disease progression [83]. Perlecan role in human squamous cell carcinoma progression may be due to recognition by its two receptors, α-dystroglycan and $\beta 1$ integrin. This association happens not only in physiological conditions [84], but also in invasive carcinoma, epithelial dysplasia, and carcinoma *in situ* [3]. Ameloblastoma presents high expression of α-dystroglycan and $\beta 1$ integrin, indicating the importance of perlecan signaling in this type of cancer as well [85].

Endorepellin has potent antiangiogenic activity [26, 86]. It was shown that antiangiogenic and antitumor growth effects of endorepellin occur due to its interaction with $\alpha 2\beta 1$ integrin [87]. It was also observed that endorepellin needs $\alpha 2\beta 1$ integrin and VEGFR2 (vascular endothelial growth factor receptor 2) to promote angiostatic activity in human umbilical vein endothelial cells (HUVECs) and porcine aortic endothelial (PAE) cells [88]. These studies may be useful in the development of strategies to delay cancer progression, since perlecan and endorepellin were shown to affect tumor angiogenesis.

4.5. Agrin. Agrin is highly expressed in carcinomas such as hepatocellular carcinoma (HCC) and cholangiocarcinoma [89–92]. It is known that agrin is capable of interacting with αV and $\beta 1$ integrins [59]. Hepatocellular carcinoma exhibits αV integrin and agrin near vessels and bile ducts, suggesting

that both molecules may promote cancer progression by increasing angiogenesis [89, 93].

4.6. Neuropilin-1. Neuropilin-1 (NRP-1) is a membrane bound HSPG that is expressed in normal tissues and in tumors like glioma, breast, colon, and pancreas. In addition, it is expressed in tumor vessels, being usually overexpressed in invasive cancers in comparison to neighboring healthy tissue. Overall, NRP-1 can be related to cancer aggressiveness [21, 94, 95]. NRP-1 is known to interact with VEGF receptor being a VEGF-dependent functional regulator [94, 95]. The presence of NRP-1 and integrins correlates with a more aggressive melanoma [96]. Melanomas which express NRP-1 become more aggressive due to the activation of αV integrin, a marker molecule in the conversion of melanoma cells to a metastatic phenotype [96]. Ruffini and colleagues found that αVβ5 integrin was involved in the transformation of cells expressing NRP-1. They have also identified a mechanism in which αVβ5 integrin inhibitor affects melanoma progression by delaying angiogenesis [96]. In this same study, it was shown that αVβ3 integrin promoted ECM invasion in the presence of VEGFR-2 in NRP-1-positive melanoma cells [96].

NRP-1 expression is increased by a glycoprotein named transmembrane NMB (GPNMB), which is known to promote malignant phenotype in breast cancer [97, 98]. GPNMB is able to bind α5β1 integrin, which activates a signaling pathway related to invasion and metastasis. Thus, GPNMB and NRP-1 must have an important role in mammary tumor growth and metastasis mediated by α5β1 integrin [98].

4.7. Betaglycan. Betaglycan, also known as TGF-β receptor type III (TβRIII), is a transmembrane proteoglycan that functions as a coreceptor for TGF-β [99, 100]. It possesses antitumoral activity by reducing cell motility and survival. In human breast cancer, TβRIII alters α5 integrin localization to sites of adhesion and the reduction of TβRIII gene expression was found to reduce overall survival in breast cancer patients. TβRIII suppresses cancer progression by stabilizing the ECM and by accumulating α5β1 integrin in its activated state; therefore, TβRIII decreased expression could disrupt ECM structure and influence α5 integrin localization, promoting cancer progression by enhancing cell motility and invasion [99].

In another study, it was shown that TβRIII knockdown decreases migratory and invasive characteristics of mesenchymal-stem-like (MSL)/triple negative breast cancer (TNBC) cells. This study shows that TβRIII knockdown is necessary to enhance α2 integrin expression, which leads to a decrease in migration and invasion of MSL/TNBC [101].

5. Closing Remarks

Integrins and heparan sulfate proteoglycans are versatile molecules that may present different functions according to the environment. Research on these molecules as agents in tumor progression is fundamental and brings to light the intricate, complex relationships occurring at cellular and subcellular levels. By analyzing HSPGs-integrin conjunct function in different types of cancer, we might be able to develop treatments based on analog molecules or develop prognostic techniques that may aid in patient treatment design. We also believe it is paramount to consider studies on other glycosaminoglycans, such as chondroitin sulfate, which may be of importance for indirect interactions with integrins.

In conclusion, we believe that as knowledge on how integrins and GAGs interact grows, our chances in succeeding to unveil mechanisms of tumor progression inhibition will be greater.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Mariana A. Soares and Felipe C. O. B. Teixeira contributed equally to this work.

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